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*STIMULATION OF MITOSIS IN ADULT MICE BY ADMINISTRATION OF THYMIDINE**

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Communicated by H. W. Magoun, April 6, 1961

Implicit in the application of tritiated thymidine to studies of cell proliferation and tissue renewal is the assumption that its administration in small doses has no pharmacodynamic effect upon subsequent cell divisions. However, a search of the literature fails to reveal any systematically derived experimental evidence in its support.

Only in isolated and somewhat specialized situations has this problem even been touched upon experimentally. For the most part, such studies have involved examination of populations either of plant cells,[1, 2] microorganisms,[3] or mammalian cell types[4–6] with the primary object of assessing tritium radiation effects rather than pharmacodynamic ones. Taken as a whole, we believe the available results have provided no conclusive evidence for or against a direct action of thymidine on dividing mammalian cells *in vivo*.

A part of the activity of this laboratory has been concerned with a comparative evaluation of mitotic indices in tissues of embryonic and adult mice. In the course of these studies, we have had occasion to derive values for the mitotic index of a given adult tissue on the basis of histological quantitation (both under normal conditions and following colchicine treatment) or by analysis of autoradiograms following administration of tritiated thymidine. Apparent discrepancies in the results of these methods have led us to postulate that tritiated thymidine might exert a stimulatory effect upon cell division when administered in doses (weights) commonly employed for *in vivo* mammalian experiments. In view of the present widespread applications of tritiated thymidine and the fundamental importance of the results obtained by its use, it appeared appropriate to inquire further into its action in the intact animal. Consequently, the experiment reported herein was undertaken, the results of which indicate that after a single injection of normal or tritiated thymidine at the 8.5–10 μg dose level, there ensues an almost immediate and prolonged stimulation of mitotic activity in the duodenum of the adult mouse.

Materials and Methods.—A total of 43 male mice of the AHE strain, 7 months old and weighing between 28 and 34 gm, were utilized in the experiment. For a month previously, the animals had been maintained in quarters controlled with respect to temperature (78–80°F) and light (13 hr darkness, 11 hr illumination per day).

Twenty-eight of the animals, constituting the experimental group, received a single dorsal subcutaneous injection of normal or tritiated thymidine. Of these, 14 received a volume of

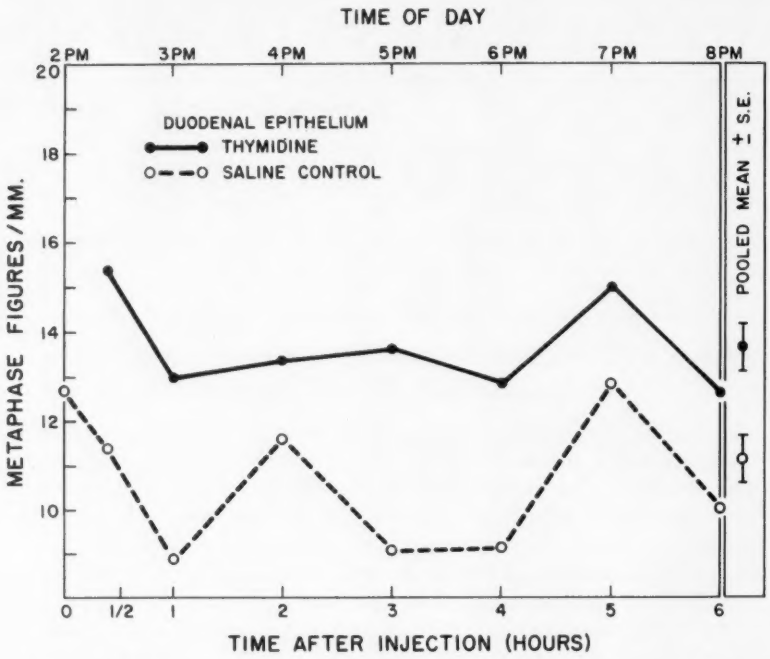

FIG. 1.—Post-injection frequency of metaphase figures plotted against time in saline-injected control animals (lower curve) and in experimental animals given a single subcutaneous injection of 8.5 μg tritiated thymidine or 10.0 μg normal thymidine (upper curve). Each experimental point (solid circle) represents the mean value of four individuals, two of which received tritiated thymidine and two of which received normal thymidine. Each control point (open circle) represents the mean value of two individuals, with the exception of the half-hour point, which is a single animal. The pooled mean values are plotted at the right with the respective standard error of their means indicated.

0.1 ml containing 10 μg normal thymidine (Schwarz BioResearch, Inc., Mount Vernon, N. Y.; Lot #TDN 6016) as a 10 mg% solution in normal saline. The remaining 14 experimental animals were given an injection of 0.05 ml saline diluted tritiated thymidine solution (Schwarz, Lot #*HTDN 1012, specific activity 0.36 curies/mM) containing approximately 8.5 μg thymidine, or approximately 12.5 μc in terms of tritium activity.

The 15 animals of the control group each received a single subcutaneous injection of 0.1 ml normal saline. The injection procedure and subsequent manipulation of the living animals were carried out in the animal quarters. Injections were given within the 15-min period from 2:00–2:15 P.M. in order to minimize diurnal differences in mitotic activity.

Two of the control animals were killed by a blow on the head immediately after injection of normal saline. At intervals thereafter of one-half, one, two, three, four, five, and six hours, animals from both control and experimental groups were killed in a similar manner. At each interval, two experimental animals which had received normal thymidine and two which had received tritiated thymidine were killed. Only one control animal was killed at the half-hour interval, whereas two control animals were killed at each subsequent interval.

Lengths of approximately 1 cm of proximal duodenum, just distal to the pyloric-duodenal junction, were removed from each animal immediately after its death. These samples were incised longitudinally, were flattened serosal side down on stiff card, and were fixed in Carnoy's fluid for 16 hr. The fixed tissues were trimmed and embedded in paraffin and oriented in the blocks so that the resultant histological sections would pass perpendicularly through the mucosa.

Four slides, each containing several 5-micron sections from different regions of the paraffin block, were prepared from each animal for histological quantitation of mitotic activity and were stained with hematoxylin-eosin. The slides were coded to minimize bias and were then examined by three observers.

The microscope of each observer was provided with an ocular reticle, which at oil immersion magnifications delimited a virtually square field of view measuring approximately 100 microns on a side. Each observer counted the number of metaphase figures visible in successive, usually adjacent, 100-micron strips of duodenal wall, including in his counts all metaphases found in the area between the base of the crypts of Lieberkühn and the villal tips. Care was exercised to count only those fields of the section which were cut perpendicular to the basal region. Portions of the sections cut in other planes, or portions in which fixation shrinkage or processing artefact was apparent, were disregarded.

Each observer counted a total of 100 metaphase figures or 100 helds (effectively 10 mm of duodenal base length) from each animal, whichever figure was last arrived at. The three sets of raw data thus obtained for each animal were then pooled and averaged and were finally expressed as the average number of metaphase figures per millimeter of duodenal base length. Ultimately, the average values for the four experimental animals at each time interval were also averaged, as were those of the pairs of control animals at each time (excepting the half-hour interval), and were plotted graphically (Fig. 1).

Additional 5-micron sections were cut from the blocks containing the tissue of the tritium thymidine-treated animals. These were prepared for autoradiography and were covered with Eastman Kodak Nuclear Track Emulsion, Type NTB2, according to the technique of Messier and Leblond.[7] Autoradiographic exposures ranged from 14 to 18 days, following which the slides were developed for two minutes in full-strength developer ("Dektol") at 18°C. After fixation and washing, the autoradiograms were strained in Harris' hematoxylin.

Results.—Analysis of variance for within-group deviation at the different time intervals (Table 1*A*) revealed no significant differences within control or experimental groups, despite the fluctuations apparent in the time plot of the averaged data (Fig. 1). Consequently, significance in the difference between the grand means of control and experimental animals was sought by the students' "*t*" test, irrespective of time of sacrifice and, in the case of the experimental animals, irrespective of whether thymidine had been administered in the normal or tritiated form.

TABLE 1

A. ANALYSIS OF DATA FROM EXPERIMENTAL AND CONTROL GROUPS FOR SIGNIFICANT VARIATION AS A FUNCTION OF TIME

Group	Variance	$\Sigma(x^2)$	*df*	Mean Square	*F*	*p*
Experimental	Between times	27.88	6	4.67	0.57	>0.05
	Within times	171.00	21	8.14		
Control	Between times	33.09	6	5.51	3.56	>0.05
	Within times	12.42	8	1.55		

B. COMPARISON OF MEANS OF POOLED EXPERIMENTAL AND CONTROL GROUPS BY THE STUDENTS' "*t*" TEST

Group	Mean	S.D.	$\Sigma(x^2)$	*df*	*t*	*p*
Pooled experimental	13.7	± 2.67	198.8	27	3.96	<0.001
Pooled control	10.6	± 1.75	46.1	14		

Reference to Table 1*B* reveals that a high degree of significance ($p < 0.001$) exists in the difference between means of the experimental and control groups. Thus, thymidine administration at the 8.5–10 μg dose level produced an increase in the size of the metaphase population in duodenal epithelium within a half-hour after injection, and in terms of mean difference, this increase averaged 29.2 per cent throughout the six-hour period of observation following administration of the drug.

Examination of autoradiograms of duodenal samples from tritium thymidine–treated animals failed to reveal any indication of abnormality in the DNA-synthetic process or in the subsequent division of labeled cells. A half-hour after injection, only interphase and some prophase cells appeared to be labeled, whereas in successive later observations, other mitotic figures were also seen to become labeled in orderly progression. After one hour, many labeled prophase and metaphase figures were observed, as well as an occasional anaphase. After two hours, labeled metaphases were very common, and although labeled anaphase and telophase figures were less frequent than metaphases, they were also very evident. By three hours, labeled cells in all stages of the mitotic cycle, as well as labeled pre- and post-mitotic cells, comprised the reactive cell population.

Discussion.—The above findings are clear-cut in their demonstration of the marked effect of a small dose of tritiated or normal thymidine upon the process of cell replication *in vivo*. A logical explanation of these findings is difficult and must in any case remain speculative pending further study. However, it seems pertinent to explore and contrast the possible toxic origin of this effect with what may be termed its physiological origin.

Possible toxic action of thymidine: It does not appear that thymidine acts as a mitotic spindle poison. If this were the case, the numbers of visible metaphase figures would tend to accumulate during the first few hours after its administration, much as in the case of colchicinized tissues.[8] Figure 1 reveals, however, that following the initial increase a half-hour after injection, the number of visible metaphases is essentially stable at the various time intervals. Our autoradiographic observations further support this conclusion in that all stages of mitosis became labeled in an orderly sequence after administration of tritiated thymidine (see below).

An alternative mechanism of toxic origin might be suggested as being responsible for increasing the number of metaphase figures. According to this, thymidine would increase the duration of the metaphase stage of mitosis or increase the duration of the entire mitotic sequence. It should be emphasized that this mechanism implies the continued availability of circulating thymidine over the six-hour period of observation. However, current evidence [9–11] concerning the rate at which exogenous thymidine is either cleared from the body or bound into newly synthesized DNA indicates that the chance of its being available in significant amounts for more than one hour after injection is very slight. Moreover, in the autoradiograms, the rapidity with which labeled interphase cells were seen to pass through prophase and metaphase to the anaphase and telophase stages of division suggests that this mechanism, if operative at all, is of minor importance.

In conclusion to this section, neither histological examination nor autoradiographic analysis has provided any indication of disturbance in structure or in the orderly sequence of events leading to cell division in the duodenal epithelium following thymidine treatment. Consequently, a basis for the increase in duodenal mitotic activity observed in the experimental animals should be sought in terms other than those of toxicity.

Possible physiological mode of action of thymidine: A wide range of dosage levels of tritiated thymidine has been utilized in prior *in vivo* mammalian experiments. In our laboratory, for example, using mice of approximately 30 gm body weight,

we have usually employed a dose of 25 μc for routine autoradiographic study of cell population kinetics. Assuming a specific activity of 0.36 curies/mM, this is equivalent to a weight of almost 17 μg of thymidine. By adapting these values to the calculations of Lajtha and Oliver,[12] it can be demonstrated that, fully utilized, this weight of thymidine would provide the entire thymidine complement of approximately 1×10^7 new cells, assuming equimolar distribution of the component nucleotides in DNA.

While it is true that thymidine utilization in DNA does not approach the 100 per cent level following injection,[11] it is equally likely that not every thymidine residue in the newly-synthesized DNA of a dividing cell would be derived from exogenous thymidine. Consequently, the actual number of cells involved may be less than, or far more than, the derived figure. In either case, any effect of exogenous thymidine would be manifested primarily in rapidly dividing populations of cells (such as the duodenal epithelium) in which DNA synthesis is frequent, and which, at any instant in time, probably represent a quite small fraction of the adult total-body cell population. As a result, the calculated number of cells potentially affected by microgram quantities of exogenous thymidine attains a considerable significance and introduces the possibility that should the effect be one of increased mitotic activity, it might be demonstrable by histological methods. This possibility appears to be borne out by the present data.

It is well established that DNA synthesis requires the simultaneous presence of its four component nucleotides.[13] Of these, presumably only thymidine nucleotide is not readily available as a free metabolite under normal circumstances, but must be formed by the methylation of deoxyuridine monophosphate in the presence of tetrahydrofolic acid.[14] Studies of dividing cell systems *in vitro* have revealed that free nucleosides added to the medium are immediately utilized for nucleic acid synthesis.[15] Folic acid antagonists have been shown to interrupt DNA synthesis in cell cultures, while the introduction of thymidine immediately brings these cells back into the DNA-synthetic phase and ultimately precipitates a marked spurt in cell division.[16]

In view of this evidence and that of the present study, we offer as a tentative hypothesis that the methylation reaction is rate-limiting in DNA synthesis *in vivo* as well as *in vitro* and can be bypassed by the addition of the finished product, i.e., thymidine. This possibility has previously been suggested and discussed by Friedkin[17] but without direct experimental support.

If such a hypothesis is correct in its major premises, it will require that figures which have been derived previously regarding the relationships of DNA-synthesis time and cell population generation time be reappraised. All of these calculations, so far as we are aware, have resulted from studies involving the use of tritiated thymidine, frequently in far larger concentration than has been employed in the present study.

Finally, our present data do not permit precise interpretation of the observed prolongation of the thymidine effect upon mitotic activity. In the light of our general hypothesis, however, it would seem likely that microgram quantities of thymidine effectively shorten DNA synthesis time *in vivo* and that the resulting increase in the number of cells entering mitosis would as a consequence be demonstrable over a period of time equal to the shortened DNA synthesis time, i.e.

approximately seven hours.[9] The detailed time relationships of this stimulation and its possible dose dependency are currently being investigated.

Summary.—Mitotic activity of the duodenal epithelium of adult male mice has been examined at intervals up to six hours after subcutaneous administration of 10 μg of normal thymidine or of 8.5 μg (12.5 μc) of tritiated thymidine. A comparison to saline-injected control animals has revealed an immediate and highly significant increase in the number of metaphase figures in the experimental animals, which was maintained throughout the six-hour period of observation and averaged 29.2 per cent during this period.

Consideration of the mechanisms which might be involved in thymidine action *in vivo* suggests that the results represent a physiological, rather than toxic, action and that the addition of free thymidine at this dose level *in vivo* shortens the average DNA synthesis time in duodenal epithelium and so permits an increase in the rate of cell entry into mitosis.

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*FURTHER STUDIES ON DEOXYRIBONUCLEIC ACID-DEPENDENT ENZYMATIC SYNTHESIS OF RIBONUCLEIC ACID**

By Debi P. Burma†, Hans Kröger‡, Severo Ochoa, Robert C. Warner, and Jacques D. Weill**

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Communicated by Severo Ochoa, April 24, 1961

A previous paper[1] described the occurrence, in *Lactobacillus arabinosus* and *Azotobacter vinelandii*, of enzymes distinct from polynucleotide phosphorylase catalyzing an incorporation of nucleotides from ribonucleoside triphosphates into RNA[2] that was markedly stimulated by DNA. It was also shown that the stimulation by DNA was not due to inhibition of nucleases present in the enzyme preparations. These observations confirmed recent reports on similar enzymes in liver nuclei,[3] *Escherichia coli*,[4, 5] and *Micrococcus lysodeikticus*.[6]

Further purification of the *L. arabinosus* and *A. vinelandii* activities yielded enzyme fractions, with decreased nuclease and RNA content, showing a pronounced dependence on the presence of GTP, UTP, and CTP, and an absolute dependence on the presence of DNA, for incorporation of ATP-C^{14} radioactivity into RNA. The activity of preparations of DNA from different sources was markedly decreased after heat denaturation suggesting that DNA is active in its native, double-stranded form.

Enzyme preparations: Ammonium sulfate fractions of *L. arabinosus* between 50 and 65% saturation were dialyzed for 6 hrs at 4° against 0.1 *M* Tris-HCl buffer, pH 7.0 (specific activity in the standard ATP-C^{14} incorporation assay,[1] with $MnCl_2$ instead of $MgCl_2$, about 1.5 mμmoles/mg protein/10 min at 37°) and treated with streptomycin sulfate at 0°. The precipitate was collected by centrifugation in the cold, dissolved in 0.1 *M* Tris-HCl buffer, pH 7.0, containing ammonium sulfate at 20% saturation, and further fractionated with ammonium sulfate. The specific activity of the 40–70% saturation fraction, used for some of the experiments described below, was about 5 but it decreased to 2 or less within a week of storage at −18°.

Dialyzed 33–46% saturation ammonium sulfate fractions of *A. vinelandii*[1] (specific activity, 1 5–2) were cooled to 0°, adjusted to pH 6.5, and treated with 1% protamine sulfate solution of the same pH until the ratio of light absorption at wave-length 280 mμ to that at 260 mμ (280/260 ratio) of the supernatant increased from about 0.6 to 0.9. The precipitate was removed by centrifugation and discarded and the supernatant shaken with a small amount of Norit for a few minutes at 0°. The supernatant solution (280/260 ratio, 1.1, about 3% RNA), with most of the activity of the ammonium sulfate fraction, had a specific activity between 3.5 and 4. The over-all purification from the initial extract was about 20-fold. The enzyme lost activity slowly on storage at −18°.

Other preparations and methods: DNA heating curves (absorbancy as a function of temperature) were obtained in the Beckman spectrophotometer with use of cylindrical cuvettes wrapped with a heating coil. Temperature was regulated with a thermistor-controlled relay.

For heat denaturation, DNA samples were dissolved (0.03–0.05 mg/ml) in 0.15 *M* sodium chloride–0.015 *M* sodium citrate, heated for 10 min at 95° (calf thymus DNA, *L. arabinosus* DNA) or 98° (*A. vinelandii* DNA) and cooled in ice. The denatured DNA was precipitated with 2 volumes of ethanol, the precipitate was dissolved in a small volume of 0.1 *M* potassium chloride and the solution dialyzed against 0.1 *M* potassium chloride for 24 hr.

Calf thymus DNA was prepared by the method of Kay *et al.*[7] as described by Chargaff.[8] Yeast RNA[9] was a gift of Dr. F. W. Allen, University of California. Other preparations and methods were as previously described.[1]

Results.—Time course: On incubation of the 33–46 ammonium sulfate fraction of *A. vinelandii* with ATP-C^{14} or GTP-C^{14} and the other three unlabeled nucleoside

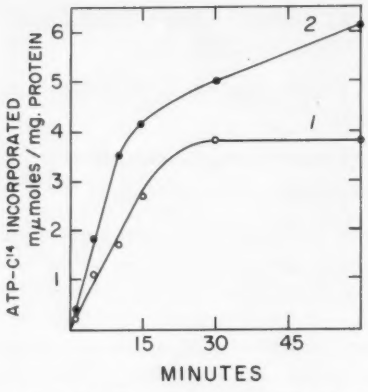

FIG. 1.—Time course of ATP-C^{14} incorporation into RNA. *Curve 1.* 1.0 ml of reaction mixture contained (in μmoles) Tris-HCl buffer, pH 8.5, 80; inorganic phosphate, 6; $MgCl_2$, 20; thioethanolamine, 1; ATP-C^{14} (3×10^5 cpm/μmole), 0.4; GTP, UTP, CTP, each 2; *A. vinelandii* DNA, 130 μg; and *A. vinelandii* enzyme with 2.2 mg protein. *Curve 2.* As curve 1 except for a higher concentration of ATP-C^{14} (2 μmoles/ml). Incubation at 37°. 0.25 ml samples pipetted into 1.0 ml of cold 5 per cent trichloroacetic acid at various time intervals.

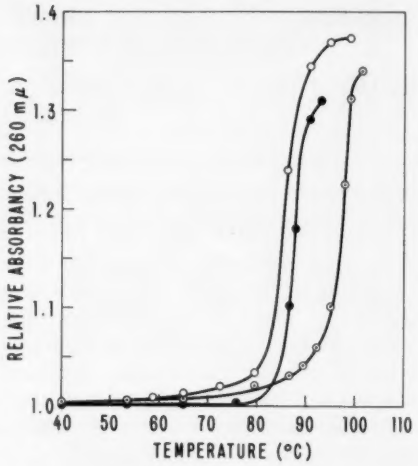

FIG. 2.—Absorbancy of DNA (wavelength, 260 mμ) as a function of temperature. DNA samples dissolved (0.017–0.025 mg/ml) in 0.15 *M* sodium chloride–0.015 *M* sodium citrate. In the range of rapid increase in absorbancy, the samples were held for 15 min at each temperature to insure attainment of equilibrium. -●-●-●-, calf thymus DNA; -○-○-○-, *L. arabinosus* DNA; ⊙-⊙-⊙, *A. vinelandii* DNA.

triphosphates there was an increase of radioactive acid-insoluble material for 10–15 min followed by a decrease.[1] With the protamine supernatant fractions (Fig. 1) the initial increase is followed either by no further change (curve 1) or by increase at a much diminished rate (curve 2) up to 60 min., presumably because of partial removal of nucleases.

Nucleoside triphosphate and DNA dependence: With the *L. arabinosus* streptomycin fraction and the *A. vinelandii* protamine supernatant the incorporation of ATP-C^{14} into RNA is markedly dependent on the presence of GTP, UTP, and CTP (Table 1). This dependence was in fact absolute with the former enzyme. As

TABLE 1

NUCLEOSIDE TRIPHOSPHATE REQUIREMENT FOR INCORPORATION OF ATP-C^{14} INTO RNA

System	Nucleotide incorporation*	
	L. arabinosus enzyme†	*A. vinelandii* enzyme‡
Complete	2.00	3.35
No GTP	<0.01	0.13
No UTP	<0.01	0.38
No CTP	<0.01	0.30
No GTP, no UTP, no CTP	<0.01	0.04
No DNA	0	0

* mμmoles/mg protein.

† Tris-HCl buffer, pH 8.0, 20 μmoles; $MnCl_2$, 5 μmoles; ATP-C^{14} (1.4×10^6 cpm/μmole), 0.1 μmole; GTP UTP, CTP, each 0.5 μmole; *L. arabinosus* DNA, 300 μg; enzyme with 90 μg protein.

‡ Tris-HCl buffer, pH 8.5, 20 μmoles; inorganic phosphate, 1.5 μmoles; $MgCl_2$, 5 μmoles; thioethanolamine, 0.25 μmole; ATP-C^{14} (3×10^5 cpm/μmole), 0.1 μmole; GTP, UTP, CTP, each 0.5 μmole; *A. vinelandii* DNA, 33 μg; enzyme with 70 μg protein. Final volume, 0.25 ml; incubation, 15 min at 37°.

TABLE 2

DNA DEPENDENCE OF ATP-C^{14} INCORPORATION INTO RNA WITH *A. vinelandii* ENZYME

Experiment no.	Additions to basic system*	Nucleotide incorporation†
1	None	0.01
	A. vinelandii DNA, 37 μg	2.93
2	None	0
	A. vinelandii DNA, 37 μg	2.52
	A. vinelandii DNA, 33 μg	2.75
3	None	0
	A. vinelandii DNA, 33 μg	2.90
	Salmon sperm DNA, 32 μg	0.72
	Yeast RNA, 48 μg	0
	A. vinelandii RNA, 49 μg	0
4	None	0
	A. vinelandii DNA, 33 μg	3.80

* Basic system: Tris-HCl buffer, pH 8.5, 20 μmoles; inorganic phosphate, 1.5 μmoles; $MgCl_2$, 5 μmoles; thioethanolamine, 0.25 μmole; ATP-C^{14} (3×10^5 cpm/μmole), 0.1 μmole; GTP, UTP, CTP each 0.5 μmole; *A vinelandii* enzyme with 0.5 mg. protein. Final volume, 0.25 ml; incubation, 10 min at 37°.
† mμmoles/mg protein.

TABLE 3

EFFECT OF NATIVE AND HEATED DNA

Experiment no.*	Enzyme (μg protein)	DNA			Nucleotide incorporation†
		Source	Condition	Amount (μg)	
1	*L. arabinosus* (90)			0	0
		L. arabinosus	Native	150	1.20
		"	"	300	1.36
		"	Heated	300	0.07
		"	Native + heated	150 each	0.53
2	*A. vinelandii* (450)			0	0
		L. arabinosus	Native	31	1.60
		"	"	62	1.95
		"	Heated	62	0.12
		"	Native + heated	62 each	0.98
3	*A. vinelandii* (210)			0	0
		A. vinelandii	Native	36	3.50
		"	Heated	36	0.75
		Calf thymus	Native	37	3.90
		"	"	65	5.20
		"	Heated	58	0.74

* Conditions in experiment 1 were the same as for the *L. arabinosus* system of Table 1. The basic system in experiments 2 and 3 was as in Table 2 except for the amount of enzyme.
† mμmoles/mg protein.

shown in the same table there was a complete dependence on the presence of added DNA in both cases. Further experiments illustrating the strict DNA dependence of the reaction with the *A. vinelandii* enzyme are shown in Table 2. RNA, on the other hand, was completely inactive (Table 2, experiment 3). The ineffectiveness of RNA is emphasized here because the previously used enzyme preparations had a high RNA content.

Effect of native and heat-denatured DNA: The effect of heating was investigated on preparations of DNA from *L. arabinosus*, *A. vinelandii*, and calf thymus. The curves of Figure 2 show the absorbancy of these nucleic acids as a function of temperature. The melting-out temperature of *A. vinelandii* DNA (97°) was significantly higher than that of *L. arabinosus* and calf thymus DNA (86° and 88°, respectively) in agreement with their A + T/G + C ratios.[10] These were found to be

0.49 and 1.27 for *A. vinelandii* and *L. arabinosus* DNA, respectively. The ratio for calf thymus DNA[8] is about 1.3. As shown in Table 3, the activity of these preparations was largely lost following denaturation by heat. It may also be seen (Table 3, last lines of experiments 1 and 2) that heat-denatured *L. arabinosus* DNA partially inhibited ATP-C^{14} incorporation brought about by native DNA. Denatured DNA may be bound by the enzyme and interfere with the binding of the active, native compound. The presence of variable amounts of denatured material in different DNA preparations might explain the observation[1] that DNA samples often inhibit nucleotide incorporation into RNA above a certain concentration.

Summary.—The incorporation of ATP-C^{14} into RNA by partially purified enzyme preparations from *Lactobacillus arabinosus* and *Azotobacter vinelandii* required the presence of each of the three triphosphates of guanosine, uridine, and cytidine and was fully dependent on the presence of native, double-stranded DNA. The three samples of DNA investigated, namely *L. arabinosus*, *A. vinelandii*, and calf thymus DNA, lost most of their activity after heat denaturation. Heat-denatured *L. arabinosus* DNA partially inhibited the incorporation brought about by the native compound.

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*ON THE ROLE OF THIAMINE PYROPHOSPHATE IN OXIDATIVE DECARBOXYLATION OF α-KETO ACIDS**

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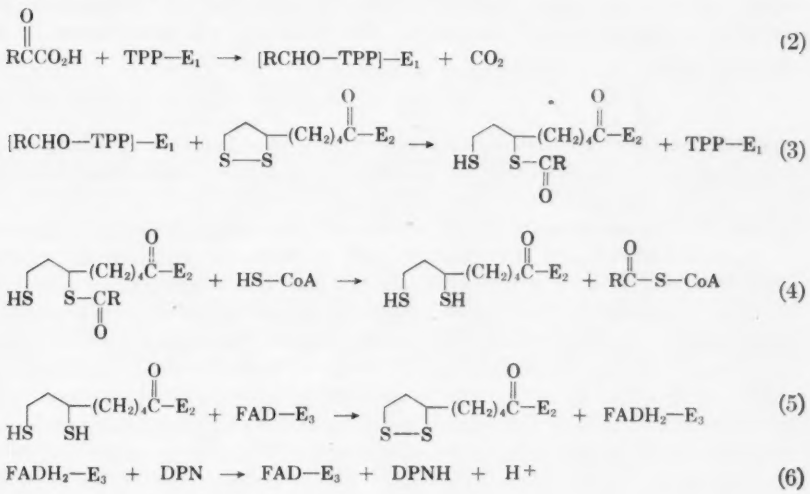
Enzyme systems which catalyze a coenzyme A- and diphosphopyridine nucleotide-linked oxidative decarboxylation of pyruvate and α-ketoglutarate (Reaction 1) have been isolated from mammalian[1, 2] and bacterial[3] cells as multifunctional

$$RCOCO_2H + CoA{-}SH + DPN \rightarrow RCO{-}S{-}CoA + CO_2 + DPNH + H^+ \quad (1)$$

complexes of high molecular weight. There is considerable evidence[4–6] that Reaction 1 proceeds via the sequence shown in Scheme 1.



Scheme 1.

Reaction 2 represents a cleavage of the α-keto acid to give carbon dioxide and an enzyme-bound "aldehyde-thiamine pyrophosphate (TPP)" compound. Reaction 3, the acyl generation reaction, has been visualized as a reductive acylation of enzyme-bound lipoic acid. Reaction 4 represents an acyl transfer to CoA, and Reaction 5 represents an oxidation of enzyme-bound dihydrolipoic acid by a flavoprotein which, in turn, is reoxidized by DPN (Reaction 6).

The pig heart α-ketoglutarate dehydrogenation complex and the *Escherichia coli* pyruvate and α-ketoglutarate dehydrogenation complexes have been separated into the flavoprotein component ($FAD{-}E_3$) and a colorless fraction which catalyzes Reactions 2–4.[7, 8] We have succeeded recently[8] in separating the *E. coli* pyruvate dehydrogenation complex into three essential components—a pyruvic carboxylase ($TPP{-}E_1$), a fraction which contains the bound lipoic acid and exhibits dihydrolipoic transacetylase activity ($lipoyl{-}E_2$), and a flavoprotein ($FAD{-}E_3$).

From the standpoint of the mechanism of thiamine pyrophosphate action, Reactions 2 and 3 of Scheme 1 are of primary interest. Breslow[9] postulated that the aldehyde moiety in the hypothetical "aldehyde-thiamine pyrophosphate" compound is linked to C-2 of the thiazole ring (cf. compound II, Scheme 2). Experimental evidence supporting this suggestion has been obtained.[10-13] In considering possible mechanisms for the acyl generation reaction (Equation 3) we were influenced by recent evidence[14] as to the site of action of ferricyanide in the oxidative decarboxylation of the respective α-keto acids catalyzed by the *E. coli* pyruvate and α-ketoglutarate dehydrogenation complexes. Previous studies[15, 16] on the stoichiometry of the latter reaction indicated that it could be represented by Equation 7.

$$RCOCO_2H + 2Fe(CN)_6^{-3} + H_2O \rightarrow RCO_2H + CO_2 + 2Fe(CN)_6^{-4} + 2H^+ \quad (7)$$

This reaction requires thiamine pyrophosphate, but neither enzyme-bound lipoic acid nor FAD is involved.[14] These latter findings suggested that ferricyanide reacts with the postulated "aldehyde-thiamine pyrophosphate" compound, presumably as represented by Equation 8. This conclusion was strengthened by the

$$[RCHO—TPP]—E_1 + 2Fe(CN)_6^{-3} + H_2O \rightarrow RCO_2H + 2Fe(CN)_6^{-4} + 2H^+ + TPP—E_1 \quad (8)$$

recent finding[8] that the carboxylase component isolated from the *E. coli* pyruvate dehydrogenation complex catalyzes Reaction 7. Reactions 3 and 8, however, presented an inconsistency. When enzyme-bound lipoic acid serves as oxidant for the "aldehyde-thiamine pyrophosphate" compound, an energy-rich acyl compound, i.e. enzyme-bound S-acyl dihydrolipoic acid, is produced (Reaction 3), whereas when ferricyanide serves as oxidant an energy-poor acyl compound, i.e. acetate or succinate, is produced (Reaction 8). Since, in the case of pyruvate oxidation, the same enzyme, i.e. pyruvic carboxylase, participates in Reactions 3 and 8, it seemed likely that a similar mechanism should obtain in both reactions. A reasonable possibility appeared to be that the "acetaldehyde-thiamine pyrophosphate" compound is oxidized in both reactions to an energy-rich acetyl compound, presumably 2-acetylthiamine pyrophosphate, which subsequently undergoes acetyl transfer to dihydrolipoyl-E_2 or hydrolysis to acetate. Recent publications[17, 18] describing the kinetic instability of 2-acylthiazolium salts point up this possibility. In the present paper experimental evidence is presented which indicates that an energy-rich acetyl compound, presumably 2-acetylthiamine pyrophosphate, is an intermediate in the ferricyanide-linked oxidation of pyruvate (Reaction 7).

In view of the low dissociability of thiamine pyrophosphate from many of the apoenzymes with which it is associated and the probable instability of 2-acetylthiamine pyrophosphate, attempts were made to trap the postulated energy-rich acetyl group rather than to accumulate 2-acetylthiamine pyrophosphate. The classical trapping agent for "active" acyl groups, hydroxylamine reagent, could not be used in Reaction 7 since this reagent reacts with ferricyanide. We have observed, however, that in the presence of dihydrogen phosphate ion the postulated acetyl group is trapped as acetyl phosphate. When Reaction 7 was carried out with the *E. coli* pyruvate dehydrogenation complex, or with the isolated carboxylase component of the complex, in the presence of phosphate buffer (pH 6.0), a substance accumulated which reacted with hydroxylamine reagent to give a hydroxamic acid.

TABLE 1

EFFECT OF PHOSPHATE CONCENTRATION ON ACETYL PHOSPHATE FORMATION*

Phosphate concentration (μmoles)	PDC		Pyruvic Carboxylase	
	Acethydroxamate (μmoles)	Ferrocyanide/2 (μmoles)	Acethydroxamate (μmoles)	Ferrocyanide/2 (μmoles)
3–5†	0	10.7	0.1	9.8
50	1.2	10.9	0.9	9.8
100	1.3	10.9	1.4	9.8
200	2.0	10.7	1.6	9.3
400	2.2	10.4	1.7	9.1
800	2.4	10.1	1.8	8.4
1400	2.6	10.1	2.2	8.4

* Each reaction mixture contained, in a final volume of 1.4 ml, 50 μmoles of potassium pyruvate, pH 6.0, 25 μmoles of potassium ferricyanide, 0.2 μmole of thiamine pyrophosphate, 0.3 μmole of $MgCl_2$, 140 μmoles of histidine buffer, pH 6.0, the indicated concentrations of potassium phosphate buffer, pH 6.0, and 1.96 mg of pyruvate dehydrogenation complex (PDC)[8] or 1.08 mg of pyruvic carboxylase.[8] The mixtures were incubated for 30 minutes at 30°. To a 1.0-ml aliquot of each incubation mixture was added 1.0 ml of hydroxylamine reagent, pH 7.0. After 10 minutes at room temperature 20 ml of 95% ethanol was added. The mixture was centrifuged, the supernatant fluid evaporated to dryness *in vacuo*, and the residue extracted with a total of 6 ml of absolute ethanol. The extract was evaporated and acethydroxamate was measured by the method of Lipmann and Tuttle.[20] Ferrocyanide in the incubation mixtures was determined as described by Hager.[16]

† The enzyme preparations contained this amount of phosphate.

TABLE 2

DETERMINATION OF ACETYL PHOSPHATE*

	Method	μmoles
1.	Hydroxamic test†	2.4
2.	(1) after heat treatment	0
3.	(1) after arsenolysis‡	0
4.	Acetokinase**	2.4

* Components and conditions were as given in legend of Table 1. 400 μmoles of phosphate buffer, but no histidine buffer, was used. At the end of the incubation period 5.6 ml of alcohol was added, the mixture was centrifuged, and the supernatant fluid was evaporated *in vacuo*.

† Acetyl phosphate was measured as described by Lipmann and Tuttle,[20] before and after heating for 2 minutes at 100°.

‡ Arsenolysis was carried out under conditions described by Stadtman.[21]

** Determined with acetokinase[22] and IDP,[23] coupled to hexokinase and Zwischenferment.[25]

The latter was identified as acethydroxamic acid by paper chromatography following the method of Stadtman and Barker.[19] The amount of acethydroxamic acid-forming material produced in Reaction 7 increased with increasing concentration of phosphate buffer (Table 1), and represented approximately 26% of the pyruvate oxidized at a phosphate concentration of 1.4 *M*. Proof that the acethydroxamic acid-forming substance was acetyl phosphate was obtained by showing that it (*a*) was heat labile, (*b*) underwent arsenolysis in the presence of arsenate, CoA, and phosphotransacetylase, and (*c*) could be coupled to a hexose phosphorylating system consisting of acetokinase, IDP, glucose, and hexokinase (Table 2). Studies on the stoichiometry of Reaction 7 showed that in the presence of dihydrogen phosphate ion pyruvate was oxidized to carbon dioxide, acetate, and acetyl phosphate (Table 3). No hydrogen or formate[27] was detected among the products, nor was formate attacked under the conditions of the reaction. These observations rule out the possibility that acetyl phosphate was produced by a phosphoroclastic-type reaction.[28] Added acetyl phosphate was recovered quantitatively from the reaction mixture. Acetyl phosphate formation was thiamine pyrophosphate-dependent and no acetyl phosphate was produced in the absence of enzyme or of ferricyanide (Table 4). The production of acetyl phosphate with ferricyanide as oxidant was not affected by removal of bound lipoic acid or of the flavoprotein from the pyruvate dehydrogenation complex.[14] This latter observation is consistent with the fact that the carboxylase component of the complex, which contains neither bound lipoic acid nor flavin,[8] catalyzes acetyl phosphate formation in the presence of ferricyanide (Tables 1, 3, 4).

TABLE 3

STOICHIOMETRY OF FERRICYANIDE-LINKED OXIDATION OF PYRUVATE*

System	Pyruvate (μmoles)	CO_2 (μmoles)	Ferrocyanide/2 (μmoles)	Acetyl-P (μmoles)	Acetate (μmoles)
PDC	−10.5	10.5	10.3	2.7	10.0
Carboxylase	−9.0	8.7	9.2	2.0	9.2

* Components and conditions were as given in legend of Table 1. 1,400 μmoles of phosphate buffer, but no histidine buffer, was used. Pyruvate was measured by a modification of the procedure of Friedemann and Haugen.[26] Acetate was determined, after hydrolysis of acetyl phosphate, with acetokinase by a modification of the method of Rose *et al.*[22]

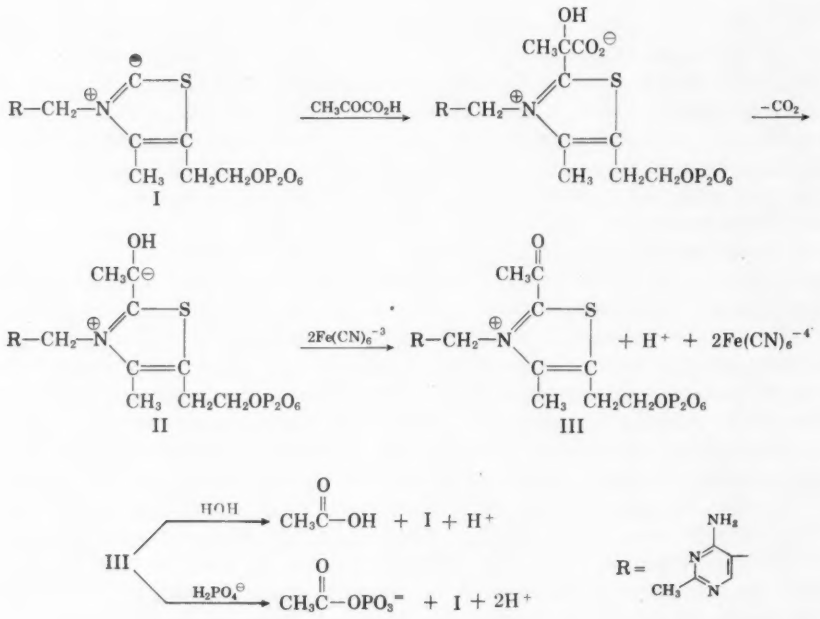
TABLE 4

REQUIREMENTS FOR ACETYL PHOSPHATE FORMATION*

System	Acetyl-P Formed	
	PDC (μmoles)	Carboxylase (μmoles)
Complete	2.6	2.2
No TPP	0.13	0
No ferricyanide	0.06	0
No enzyme	0	0

* Components and conditions were as given in legend of Table 1. 1,400 μmoles of phosphate buffer, pH 6.0, was used.

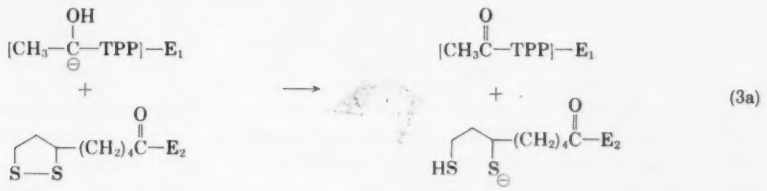
The formation of acetyl phosphate in the ferricyanide-linked oxidation of pyruvate strongly indicates that an energy-rich compound, presumably 2-acetylthiamine pyrophosphate, is an intermediate in the reaction. The results are consistent with the formulation of the reaction shown in Scheme 2, which is based on the mechanism of thiamine action in model systems advanced by Breslow.[10]



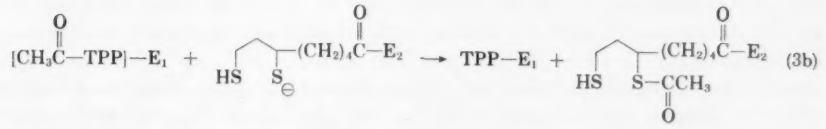
SCHEME 2.

Nucleophilic attack of water or dihydrogen phosphate ion on the carbonyl carbon atom of 2-acetylthiamine pyrophosphate (III) would produce acetic acid and acetyl phosphate, respectively. Presumably these are competing reactions since the proportion of pyruvate converted to acetyl phosphate increases with increasing concentration of phosphate buffer (cf. Table 1).

The possibility exists that 2-acetylthiamine pyrophosphate is an intermediate in the oxidative decarboxylation of pyruvate involving the physiological oxidant, enzyme-bound lipoic acid (Reaction 3, Scheme 1). The postulated two-step reaction can be represented as:



(3a)



(3b)

The high degree of spatial orientation of proteins and coenzymes which must exist in the pyruvate dehydrogenation complex would favor Reaction 3b at the expense of the hydrolytic reaction (cf. Scheme 2).

The ferricyanide-linked oxidation of α-ketoglutarate (Reaction 7) catalyzed by the *E. coli* α-ketoglutarate dehydrogenation complex produced, in the presence of phosphate buffer (pH 6.0), a small amount of hydroxamic acid-forming material. The hydroxamic acid was identified as succinohydroxamic acid by ion exchange chromatography[29] followed by paper chromatography.[30] The succinohydroxamic acid-forming material was heat labile and presumably was succinyl phosphate. Further identification of this material is in progress. The amount of succinohydroxamic acid-forming material produced in Reaction 7 increased with increasing concentration of phosphate buffer (Table 5). However, the maximum amount of succinohydroxamic acid obtained was only 18 per cent of the amount of acethydroxamic acid obtained from the oxidation of pyruvate by the pyruvate dehydrogenation complex under similar conditions (cf. Table 1). A possible explanation of this difference is that steric hindrance and/or electrostatic repulsion impedes the

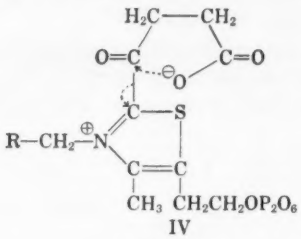
TABLE 5

EFFECT OF PHOSPHATE CONCENTRATION ON SUCCINYL PHOSPHATE FORMATION*

Phosphate concentration (μmoles)	Succinohydroxamate (μmoles)
200	0.10
400	0.26
800	0.46
1400	0.46

* 50 μmoles of potassium α-ketoglutarate and 0.69 mg of *E. coli* α-ketoglutarate dehydrogenation complex were used. Other components and conditions were as given in legend of Table 1, with the exception that histidine buffer was not present. The ferricyanide (25 μmoles) was completely reduced in 12 minutes.

attack of the relatively large dihydrogen phosphate ion on the α-carbonyl carbon atom of the hypothetical 2-succinylthiamine pyrophosphate (IV). In contrast to 2-acetylthiamine pyrophosphate (III), 2-succinylthiamine pyrophosphate contains a



IV

negatively charged acyl group. It is also conceivable that the latter substance undergoes an intramolecular attack of the carboxylate ion on the α-carbonyl carbon atom to give succinic anhydride, which would undergo rapid hydrolysis to succinic acid in the aqueous medium (cf. ref. 31). The latter possibility would provide a reasonable explanation for the observation[14] that the rate of Reaction 7 as catalyzed by the α-ketoglutarate dehydrogenation complex with α-ketoglutarate as substrate is approximately 10 times the rate obtained with the pyruvate dehydrogenation complex and pyruvate as substrate. Resolution of the α-ketoglutarate dehydrogenation complex and comparison of the two carboxylases should shed further light on this problem.

Summary.—Evidence is presented which indicates that an energy-rich acetyl compound, presumably 2-acetylthiamine pyrophosphate, is an intermediate in the ferricyanide-linked oxidation of pyruvate catalyzed by either the *E. coli* pyruvate dehydrogenation complex or pyruvic carboxylase isolated from the complex. An energy-rich succinyl compound, presumably 2-succinylthiamine pyrophosphate, appears to be an intermediate in the ferricyanide-linked oxidation of α-ketoglutarate catalyzed by the *E. coli* α-ketoglutarate dehydrogenation complex.

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† Rosalie B. Hite Postdoctoral Fellow, 1960–1961.

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*THE MECHANISM OF PEPSIN ACTION**

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The initial studies on the action of crystalline swine pepsin on synthetic substrates showed that this enzyme hydrolyzes preferentially peptide bonds involving the imino group of L-tyrosine or of L-phenylalanine,[1] in contrast to the action of crystalline beef chymotrypsin, which hydrolyzes preferentially bonds involving the carbonyl group of an aromatic amino acid residue. The subsequent demonstration that chymotrypsin[2] and other proteinases[3, 4] catalyze transamidation reactions in which an acyl group is transferred from a substrate to an acceptor amine was consistent with the formulation of the mechanism of their action as a "carbonyl transfer,"[5] in which an intermediate "acyl-enzyme" is involved.[6] Recent studies[7] have provided strong evidence for the view that, in the case of chymotrypsin, the acyl group is attached to the β-hydroxyl group of a serine residue in the enzyme protein.

In a recent publication, Neumann *et al.*[8] have shown that when pepsin is incubated with a synthetic substrate such as carbobenzoxy-L-tyrosyl-L-tyrosine, there is formed L-tyrosyl-L-tyrosine in amounts that can be detected by means of paper chromatography or of ionophoresis. These investigators have interpreted their results as indicating the occurrence of "imino transfer" in the catalytic action of pepsin:

(1) $RCO\text{-}NHR' + \text{Pepsin-OH} \rightleftarrows RCOOH + \text{Pepsin-}NHR'$
(2) $\text{Pepsin-}NHR' + H_2O + H^+ \rightarrow R'NH_3^+ + \text{Pepsin-OH}$ (Hydrolysis)
(3) $\text{Pepsin-}NHR' + R''COOH \rightleftarrows R''CO\text{-}NHR' + \text{Pepsin-OH}$ (Transfer)

The present communication reports additional evidence, based on an isotope exchange experiment, in favor of the occurrence of reaction (1) in the pepsin-catalyzed cleavage of peptide bonds. The experiment involved three incubation solutions whose composition was as follows: Solution A (10 ml) contained 50 micromoles each of carbobenzoxy-L-tyrosyl-L-tyrosine,[9] of carbobenzoxy-L-tyrosine,[10] and of C^{14}-L-tyrosine (2700 cpm per micromole; uniformly labeled C^{14}-L-tyrosine from Nuclear-Chicago diluted with unlabeled tyrosine), 10 mg of crystalline swine pepsin (Armour), and 0.1 *M* acetate buffer (pH 4.0). The composition of solution B was similar to that of solution A, except that the carbobenzoxy-L-tyrosine was labeled (2,700 cpm per micromole) and the L-tyrosine was not. Solution C was a nonenzymic control for solution B, and the pepsin was omitted from the incubation mixture.

The three solutions were kept at 37.5°C for 22 hours. A separate experiment, using unlabeled compounds, showed that during this time period, the enzyme caused an increase in ninhydrin-reactive material[11] corresponding to 17 per cent hydrolysis of carbobenzoxy-L-tyrosyl-L-tyrosine at one amide bond.

After the incubation period, 150 ml of ethanol were added to each of the three solutions, the mixtures were chilled overnight, and filtered. The three filtrates were worked up in identical manner; each of them was concentrated to dryness under reduced pressure, and the residue was extracted with methanol. The carbobenzoxy compounds present in the methanolic solution were subjected to catalytic hydrogenolysis with palladium black in the usual manner; about 60 minutes were required. After removal of the catalyst by filtration, the methanolic solution was concentrated to dryness under reduced pressure, and the residue was dissolved in a small volume of water. Paper chromatography (Whatman No. 1 paper) with *n*-butanol-acetic acid-water (4 : 1 : 5) as the solvent showed the presence of two ninhydrin-positive components, having R_F values of 0.40–0.42 and 0.60–0.64, identical with those given by authentic samples of L-tyrosine and of L-tyrosyl-L-tyrosine respectively. The component having R_F 0.60–0.64 (L-tyrosyl-L-tyrosine) was eluted, and purified by subjecting it twice to large-scale paper chromatography, followed by elution with water. To an aqueous solution of each of the three samples of L-tyrosyl-L-tyrosine obtained in this manner, there was added 50 mg of an authentic sample of the unlabeled dipeptide, the solution was concentrated to dryness, and the residue was dissolved in ethanol. Upon the addition of ether, the tyrosyltyrosine crystallized, and each sample was recrystallized once more. Their specific radioactivity was determined by means of gas-flow counter (background 10 ± 1 cpm) with 1.8–2.2 mg of sample per cm^2 on the planchet. The dipeptide samples from solution A (with C^{14}-labeled tyrosine) and from solution C (no pepsin, with C^{14}-labeled carbobenzoxy-L-tyrosine) gave no counts over background, whereas the dipeptide sample from solution B (complete system, with labeled carbobenzoxy-L-tyrosine) had a specific radioactivity of 22 cpm per mg. The last value was unchanged (within the precision of the counting procedure) after an additional recrystallization of the L-tyrosyl-L-tyrosine from Solution B. Since 50 mg of unlabeled carrier had been added after chromatographic separation of the dipeptide from the hydrogenated incubated mixture, it may be estimated that there were about 1,100 cpm of C^{14} in labeled L-tyrosyl-L-tyrosine. This amount of exchange of labeled carbobenzoxy-L-tyrosine into the carbobenzoxy dipeptide is clearly a

minimal value, since losses of the labeled dipeptide must have been incurred during the purification by paper chromatography.

The L-tyrosyl-L-tyrosine from solution B was treated with 1-fluoro-2,4-dinitrobenzene to yield the DNP-peptide, which was subjected to hydrolysis with 6 *N* HCl for 12 hours at 110°C. Extraction of the hydrolysate with ether gave a fraction that retained about 90 per cent of the radioactivity of the dipeptide, indicating that the dipeptide had been labeled in the amino-terminal tyrosyl residue, and that the carboxyl-terminal residue was unlabeled.

It should be noted that any L-tyrosyl-L-tyrosine formed by the reaction of the two molecules of the carbobenzoxy dipeptide to give the carbobenzoxy tripeptide, followed by the hydrolysis of the last named compound, would be expected to yield only unlabeled dipeptide. Furthermore, since carbobenzoxy-L-tyrosine is resistant to the action of pepsin under the conditions of the above experiment, the appearance of C^{14} in the L-tyrosyl-L-tyrosine from solution B must involve the reaction of carbobenzoxy-L-tyrosine as a unit. The simplest explanation is the occurrence of the exchange reaction (1). The fact that the dipeptide from solution A was not radioactive makes an acyl-transfer reaction unlikely in the case of pepsin. The results of the isotope exchange experiment described above are therefore in accord with the specificity studies[1] and transfer experiments[8] in favoring a mechanism of pepsin action that involves activation of the imino group of the sensitive peptide bond.

The unique properties of pepsin—its very acid isoelectric point, its ready denaturation at pH values above 6, its relatively high content of the residues of dicarboxylic amino acids, β-hydroxy amino acids, aromatic amino acids, and proline—invite speculation about the nature of the group (or groups) in the protein that are involved in its catalytic action. It has been suggested[12] that a protein carboxyl group of abnormally low pK may accept the NH group of the sensitive linkage to form an amide bond, which is cleaved either by water (hydrolysis) or by a carboxylic acid (transfer). The report[13] that the single phosphoryl group (per unit of about 35,000) of pepsin may be removed enzymically without affecting pepsin activity appears to rule out such a group as a participant in the catalytic action. Clearly, further studies, both with pepsin itself and with model compounds that may mimic its action, are needed to clarify the mechanism of pepsin action.

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*ASSOCIATION OF H-2 ANTIGENS WITH THE CELL MEMBRANE FRACTION OF MOUSE LIVER**

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Of the fifteen or more genetic loci in mice that determine antigens capable of eliciting homograft rejection,[1] only one, the H-2, has been shown also to control erythrocyte antigens detectable by hemagglutination.[2] It recently has become feasible, with the development by Stimpfling of an easy, reproducible hemagglutination method for mouse red blood cells,[3] to use the absorption of hemagglutinins as an index of H-2 antigen throughout a purification procedure.

Using this method, we demonstrate below the isolation of most of the H-2 antigenic activity of mouse liver in a lipid- and protein-containing fraction which appears to consist principally of nuclear and cellular membranes.

Materials and Methods.—Mouse strains:

DBA/2J	$H\text{-}2^d$	Retired breeders via Simonsen Laboratories, Gilroy, California.
C3H/Bi	$H\text{-}2^k$	From Simonsen Laboratories.
C57Bl/6J	$H\text{-}2^b$	Retired breeder females from incross, Jackson Memorial Laboratories, Bar Harbor, Maine.
C57Bl/10J	$H\text{-}2^b$	From Jackson Memorial Laboratories.
C57Bl/10-H-2^d (B10.D2)	$H\text{-}2^d$	Courtesy of Dr. G. D. Snell.
DBA/2JTu	$H\text{-}2^d$	From the colony of the late Dr. J. W. Turner.

Solutions: Phosphate buffered saline (PBS) contains 7.65 gm NaCl, 0.725 gm Na_2HPO_4, and 0.212 gm KH_2PO_4 in 1 liter distilled water, pH adjusted to 6.5.

PVP-PBS contains 15 gm polyvinylpyrrolidone (PVP) (33 gm of 45% aqueous solution of type NP-K60 obtained from Antara Chemicals, Division of General Aniline Corporation) in 1 liter PBS. It was autoclaved and stored at room temperature.

Sucrose-phosphate contains 85.8 gm sucrose per liter $M/75$ phosphate buffer pH 7.0 (Sorensen's phosphate buffer diluted $^1/_5$).

Citrate saline contains 1 part of 3.4% sodium citrate to 4 parts 0.85% NaCl.

Red blood cells: Red blood cells from the appropriate mouse strain were collected in citrate-saline, washed 3 times with 0.85% NaCl, resuspended in PBS to 10% and stored in the refrigerator. Before use, a further dilution to 2% with PBS was made.

Hemagglutination: The method of hemagglutination used was a slight modification of the method developed by Stimpfling.[3] Each 10 mm tube contained 0.1 ml of a serial dilution of antiserum in PVP-PBS plus 0.05 ml of 2% red blood cell suspension in PBS. After 30 minutes, the tubes were centrifuged for 30 seconds at approximately $250 \times g$, 0.85% NaCl (0.3–0.5 ml) was layered over the PVP-PBS, and then, with a Pasteur pipette, a jet was gently directed at the pellet. If agglutination occurred, the cells came up as: one large clump (scored as 4), several large clumps (scored as 3), small clumps and loose cells (scored as 2), or mainly loose cells with some persistent clumps (scored as 1). In the absence of agglutination, there was a cloud of loose cells, no clumps (scored as negative). The titer was taken as the last serum dilution in the series to be scored as 1 or more.

Absorption: Material to be used to absorb antiserum was packed by centrifugation, then resuspended in antiserum diluted $^1/_{20}$ in PVP-PBS. After 30 minutes at 8°C, the absorbing material was once again centrifuged, this time in the cold, and the antiserum was removed and titrated.

Antiserum production: $H\text{-}2^b$ (C57Bl/6J) female breeders (retired from incrossing) were injected with DBA/2JTu cell suspension six times at weekly intervals, (1 spleen and thymus per 30

animals per injection). Blood was collected from the tail artery, pooled, and allowed to clot overnight in the refrigerator. The clot was removed and small aliquots of serum were frozen in sealed ampoules.

No diminution of titer of antiserum has been noted due to freezing or storage for over 6 months at −20°C.

Protein determinations: Protein was determined by the method of Lowry *et al.*[4] Crystalline bovine serum albumin (Armour) was used as standard.

Experimental Results.—Isolation: Livers from exsanguinated H-2^d (DBA/2J) and H-2^k (C3H/Bi) mice were ground with a Duall[5] homogenizer in 5 volumes of sucrose-phosphate and centrifuged for 3 minutes at 250 × *g* (Fig. 1). The sediment, consisting mainly of nuclei, cell membranes and debris, was resuspended in

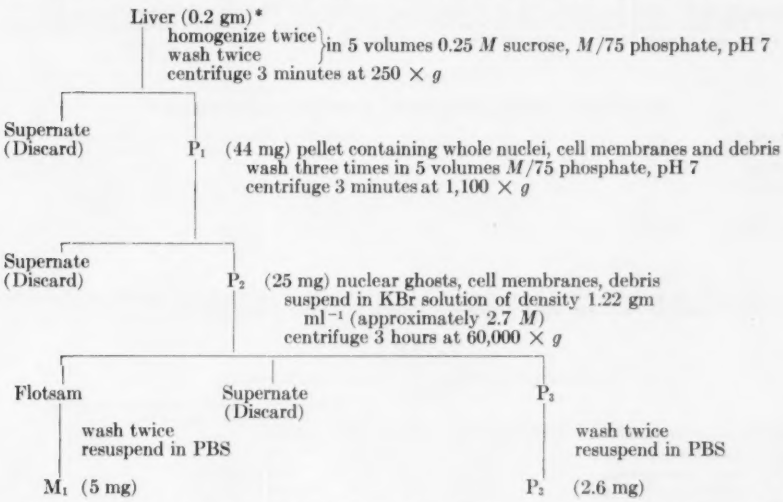
Liver (0.2 gm)*
homogenize twice
wash twice
centrifuge 3 minutes at 250 × g
in 5 volumes 0.25 M sucrose, M/75 phosphate, pH 7
Supernate
(Discard)
P1 (44 mg) pellet containing whole nuclei, cell membranes and debris
wash three times in 5 volumes M/75 phosphate, pH 7
centrifuge 3 minutes at 1,100 × g
Supernate
(Discard)
P2 (25 mg) nuclear ghosts, cell membranes, debris
suspend in KBr solution of density 1.22 gm ml−1 (approximately 2.7 M)
centrifuge 3 hours at 60,000 × g
Flotsam
Supernate
(Discard)
P3
wash twice
resuspend in PBS
wash twice
resuspend in PBS
M1 (5 mg)
P3 (2.6 mg)

FIG. 1.—Isolation flow sheet. * The figures in parentheses give protein content expressed per gm fresh weight of liver.

sucrose-phosphate, homogenized again and washed twice (P_1). It was then suspended in *M*/75 phosphate buffer, pH 7.0 (which lysed the nuclei), centrifuged for 3 min at 1100 × *g* and washed twice in the same buffer (P_2). Next it was suspended in potassium bromide solution at a final density of 1.22 gm ml^{-1} (approximately 2.7 *M*, or 317 gm KBr per liter solution) and centrifuged 3 hr at 60,000 × *g*.

After centrifugation the material in the tube was distributed as (1) a compacted sheet of flotsam at the top, which was lifted off with a 60-gage stainless-steel-screen spatula, washed twice, and resuspended in PBS (M_1), (2) a clear viscous supernatant which was discarded, and (3) a pellet, which was washed twice and resuspended in PBS (P_3). The M_1 fraction contained 2 per cent of the protein of the original liver homogenate.

After several hours in the refrigerator, M_1 expanded and became fluffy. When observed microscopically, it appeared to consist mostly of cellular and nuclear membranes.

Immunological characterization: Incubation of 4.0 mg M_1 with 0.2 ml of a $^1/_{20}$ dilution of an H-2^b anti H-2^d antiserum, which has a titer of $^1/_{1280}$ to $^1/_{2560}$, for 30

TABLE 1

ABSORPTION OF HEMAGGLUTININS BY MEMBRANE FRACTION*

M_1 (Membrane fraction) Mg Protein	Antiserum dilution (after absorption) (1/20)	(1/40)	(1/80)	(1/160)	(1/320)	(1/640)	(1/1280)	(1/2560)
4.0	†1	1	±	−	−	−	−	−
3.0	1	1	1	−	−	−	−	−
2.0	2	3	2	±	−	−	−	−
1.0	2	4	2	2	1	−	−	−
0.5	3	3	2	2	1	−	−	−
0.25	2	4	4	4	3	2	1	−
Unabsorbed antiserum	4	3	3	2	3	3	2	1
Normal serum	−	−	−	−	−	−	−	−

* Antiserum used: H-2^b anti H-2^d. 0.2 ml of serum was absorbed at 1/20 dilution for 30 minutes at 8°C.
† 4, 3, 2, 1 indicate agglutination, 4 being the greatest, 1 being the weakest; ± indicates questionable agglutination; − indicates no agglutination. Also see *Materials and Methods* for further explanation.

TABLE 2

ANTI H-2^d ACTIVITY OF C57Bl/6J ANTI-DBA/2JTu SERUM

Source of erythrocytes	H-2 allele	Antiserum dilution (1/40)	(1/80)	(1/160)	(1/320)	(1/640)	(1/1280)	(1/2560)
C57Bl/ 10-H-2^d	H-2^d	*4	4	4	2	1	1	−
C57Bl/10J	H-2^b	−	−	−	−	−	−	−

* 4, 3, 2, 1 indicate agglutination, 4 being greatest, 1 being weakest; ± indicates questionable agglutination; — indicates no agglutination. Also see *Materials and Methods* for further explanation.

TABLE 3

SPECIFICITY OF HEMAGGLUTININ ABSORPTION*

Material used to absorb	Equiv. gm fresh wt. liver	Reciprocal of hemagglutination titer with erythrocytes from H-2^d	H-2^k
H-2^d P_2 (lysed homogenate)	1.0	40	−†
H-2^b P_2 (lysed homogenate)	1.0	640	−
H-2^d M_1 (membrane fraction)	1.0	<40	<40
H-2^k M_1 (membrane fraction)	1.0	160	<40
Unabsorbed serum		640	320

* Antiserum used: H-2^b anti H-2^d. 0.2 ml of serum was absorbed at 1/20 dilution for 30 minutes at 8°C.
† (−) indicates not tested.

minutes at 8°C reduced the titer of the serum to 1/40 (Table 1). (The unabsorbed serum contains anti H-2^d antibodies as it agglutinates erythrocytes from C57Bl/10-H-2^d but does not agglutinate erythrocytes from the coisogenic strain C57Bl/10 (H-2^b) (see Table 2). The decrease in titer is roughly proportional to the amount of antigen used over the range 4.0 to 0.25 mg protein (Table 1).

The specificity of absorption was demonstrated in two ways. A fraction (P_2) prepared from H-2^d livers removed nearly all the anti H-2^d activity from the serum, while a similar fraction prepared from H-2^b liver (the strain of the serum donor) left the hemagglutinins intact (Table 3, lines 1 and 2).

A more critical test of specificity was based on the selective absorption of antibodies from a serum, utilizing the immunological cross-reactivities of H-2 antigens (Table 4). Thus, an anti H-2^d serum prepared in H-2^b animals would be expected to cross-react with antigens in the H-2^k strain, and therefore to agglutinate H-2^k erythrocytes. An H-2^k fraction should remove hemagglutinins directed against the cross-reacting components (C, H) of H-2^k but should leave behind the hemag-

TABLE 4

RELEVANT H-2 COMPONENTS AND EXPECTATION OF ANTIBODY SPECIFICITIES[6]

Serum	Strain	H-2 allele		Relevant antigenic components								Erythrocytes agglutinated
	C57Bl/6	$H\text{-}2^b$		–	D^b	E	F	–	–	–	N	Z	
	DBA/2	$H\text{-}2^d$		C	D	E^d	F	H	J	M	N	Z	
$H\text{-}2^b$ anti $H\text{-}2^d$			*anti*	*C*	*D*	*E^d*	–	*H*	*J*	*M*	–	–	$H\text{-}2^d$, $H\text{-}2^k$
	C3H	$H\text{-}2^k$		C	–	–	–	H	–	–	–	–	
$H\text{-}2^b$ anti $H\text{-}2^d$ absorbed with $H\text{-}2^k$			*anti*	–	*D*	*E^d*	–	–	*J*	*M*	–	–	$H\text{-}2^d$

TABLE 5

RECOVERY OF HEMAGGLUTININ ABSORBING ACTIVITY DURING FRACTIONATION*

Fraction used to absorb	Equivalent fresh weight of liver (gm)	Protein (mg)	Antiserum dilution (after absorption)							
			(1/20)	(1/40)	(1/80)	(1/160)	(1/320)	(1/640)	(1/1280)	(1/2560)
P_1 (homogenate before lysis)	0.6	26	†±	–	–	–	±	–	–	–
P_2 (lysed homogenate)	0.6	15	±	–	–	–	–	–	–	–
S_1 (KBr sediment)	0.6	1.6	2	2	2	2	2	±	–	–
M_1 (membrane fraction)	0.6	3.0	1	1	1	–	–	–	–	–
Unabsorbed serum	—	–	4	3	3	2	3	3	2	1

* Antiserum: $H\text{-}2^b$ anti $H\text{-}2^d$. 0.2 ml of serum was absorbed at 1/20 dilution for 30 minutes at 8°C.
† Same as † footnote in Table 1.

TABLE 6

ANALYSIS OF MEMBRANE FRACTION

Material	Dry weight (mg)	Protein (mg)	Carbohydrate (mg)[7] (α-Napthol)	(Indole)	Hexosamine[8] (mg)
M_1 total	6.45	4.95	0.05	0.04	<0.01
Lipid soluble[9]	2.65*	0.55	–	–	–
Lipid insoluble[9]	3.80	3.77	–	–	–

* Figures indicate mg per 6.45 mg dry weight of M_1. Each value is the average of 2 determinations. 60–80 mg dry weight of M_1 is a usual yield from 10 gm fresh weight of liver.

glutinins directed against the remaining $H\text{-}2^d$ components. $H\text{-}2^d$ M_1 should, of course, remove all the hemagglutinins.

The results conformed to the above predictions when the appropriate absorptions were performed (see Table 3, lines 3 and 4). Absorption of the $H\text{-}2^b$ anti $H\text{-}2^d$ serum with $H\text{-}2^d$ M_1 removed all hemagglutinating antibody (including that which could have agglutinated $H\text{-}2^k$ erythrocytes). Absorption with $H\text{-}2^k$ M_1 removed only the cross-reacting antibodies, removing therefore all the titer against $H\text{-}2^k$ erythrocytes but leaving a substantial titer against $H\text{-}2^d$ erythrocytes.

In Table 5, the recovery of H-2 antigen throughout the purification procedure is estimated. Most of the activity is recovered in the M_1 fraction. Reworking of the sediment (P_2) gave additional active M_1 fraction and an inactive sediment.

Chemical characterization: As has been pointed out earlier, the M_1 fraction consists principally of cellular and nuclear membranes, and therefore is most decidedly not pure antigen. However, chemical characterization of the fraction is of interest with respect to both the chemistry of membranes and the chemical nature of the H-2 antigens.

The material in M_1 is almost entirely lipid and protein (Table 6). There is less than 1 per cent total carbohydrate, and hexosamine is not detectable.

The antigenic activity is stable for a week or more in the refrigerator but was not detectable after exposure to 56–60°C for 1 hr or 100°C for 5 min.

Discussion.—Serological methods as assays for transplant antigens must be used cautiously due to the uncertainty that antigens which react with hemagglutinins are identical to those which elicit a transplantation immunity.[10, 11] However, the evidence that hemagglutinins are also enhancing antibodies,[12] which act by blocking the passage of antigens from a graft to the sites where they can evoke the cellular immune response,[13] encourages the use of methods involving humoral antibodies for isolation of transplantation antigens, even though a material cannot be finally accepted as a transplant antigen until its injection can be shown to cause an accelerated homograft reaction.

Using hemagglutinin absorption as an index of antigen offers certain advantages in the mouse beyond the rapidity with which tests can be made. It immediately restricts consideration to the antigen (or antigens) controlled by the H-2 locus, as agglutination of erythrocytes by isoantisera other than those directed against H-2 antigen (or antigens) is not readily demonstrable.[14] This permits the use of standard mouse stains such as C57Bl/6 and DBA/2 which differ at a number of histocompatibility loci without losing the selectivity offered by Snell's (less readily available) isogenic resistant pairs, which differ only at a single locus.

Histocompatibility antigens have been found in the nuclear, mitochondrial and microsomal fractions.[15, 16] This is not inconsistent with our finding of most of the H-2 antigen in the cell membranes, since nuclear fractions usually contain cell membranes; and when cells are disrupted by sonic oscillation, membrane fragments become sedimentable only by high-speed centrifugation[17] and will thus contaminate the mitochondrial and microsomal fractions.

Most of the antigen present in the extract after the first homogenization is recovered in the fraction which on centrifugation floats in the high-density potassium bromide solution. This material was observed microscopically to consist of folded, membranous structures suggestive in appearance of empty liver parenchymal cells. Electron microscope studies on a rat liver membrane preparation obtained using a similar fractionation scheme[18] (which differed from our own essentially in the use of high-density sucrose solution instead of a potassium bromide solution for the flotation step) showed that the structures isolated are nuclear and cell membranes.

The amino sugar and carbohydrate content of our preparation is lower than that reported by Kandutsch and Reinert-Wenck[16] for their preparations of enhancement factor, but as the data do not permit an estimation of the percentage of H-2 material in a fraction, the small carbohydrate content could still be a major constituent of the H-2 component. In fact, as long as the antigen resides in an insoluble cell fraction, where its purity cannot be determined, conclusions based on stability to treatment with various agents (e.g. heat, solvents, enzymes) must be constantly under review. Any effect may be due either to a change in the antigen or to a change in the material surrounding the antigen, rendering the antigenic activity undetectable.

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THE SOLUBILIZATION OF MICROSOMAL ANTIBODY ACTIVITY BY THE SPECIFIC INTERACTION BETWEEN THE CRYSTALLIZABLE FRACTION OF γ-GLOBULIN AND LYMPH-NODE MICROSOMES

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In a previous study,[1] it was shown that when 2,4-dinitrophenyl bovine γ-globulin is injected into guinea pigs there appears in the microsomal fraction of regional lymph nodes an antibody activity which is specific for the 2,4-dinitrophenyl group. The present work is concerned with the questions: (1) is the antibody activity of lymph-node microsomes[2] due to conventional serum antibody linked to microsomes, and, if so, (2) what kind of linkages are responsible? In order to examine these questions it was necessary to determine the conditions required to separate antibody activity from microsomes and to compare the solubilized material with serum antibody of the same specificity.

The recent work of Porter[3] describing three fractions of rabbit γ-globulin and antibodies, obtained by papain digestion and chromatography, has led to a significant advance in understanding the structure of antibodies and has provided methods which may prove applicable to studies of antibody synthesis. In the present report, evidence is presented that microsomal antibody activity is the result of a specific association between lymph-node microsomes and that segment of γ-globulin which corresponds to Porter's fraction III.

Materials and Methods.—The materials used and the procedures followed for immunization of guinea pigs and preparation of lymph-node microsomes have been described.[1] In principle, the method used for measuring microsomal anti-DNP[4] antibody activity involves incubation of the microsomal fraction with an indicator antigen (I^{131}-DNP-BSA), washing the particles by suspension and sedimentation (105,000 × *g*), and, finally, elution of the radioactive indicator antigen from the microsomal pellet with a homologous hapten (ε-DNP lysine; see Table 1). This assay, which was described previously in detail,[1] has been simplified in the present work by eliminating one of the two washing procedures (step 3, Fig. 1 of ref. 1) and by eliminating bovine serum albumin from the medium used for washing and eluting the microsomes (steps 2 and 4 of Fig. 1, ref. 1). The basic medium used for homogenization of lymph nodes and for isolation and assay of microsomes was medium A of Littlefield and Keller:[5] 0.35 *M* sucrose, 0.05 *M* Tris, pH 7.6, 0.004 *M* $MgCl_2$, and 0.025 *M* KCl. Microsomal protein was isolated by the method of Schneider,[6] dissolved in 5.2 *M* acetic acid, and measured by its absorbance at 280 mμ; its extinction coefficient, $E^{1\%}_{1cm}$, is 10, not 13.6 as previously given.[1,2] In experiments in which mitochondria and microsomes were compared, these 2 fractions were isolated from the same lymph-node homogenate (0.25 *M* sucrose) by the method used for rat liver.[7] The mitochondria were washed 3 times to reduce contamination by microsomes.

Fractions of rabbit γ-globulin (I, II, and III) were prepared and isolated as described by Porter.[3]

H^3-labeled antibody, specific for the 2,4-dinitrophenyl group, was prepared biosynthetically by incubating 4,5-H^3-DL-leucine with lymph-node cells that were isolated from guinea pigs immunized with DNP-BγG. The procedures for carrying out the incubation, for isolation of H^3-anti-DNP antibody and for preparing a particulate fraction from the incubated cells are given elsewhere.[8]

For some control experiments, lymph-node microsomes were isolated from guinea pigs immunized with an antigen lacking the DNP determinant. The latter antigen was prepared by coupling BγG with diazotized *p*-arsanilic acid; the conjugate (R-azo-BγG) was purified by alternately precipitating at pH 4 and dissolving at pH 8.[9] Guinea pigs were injected with 0.8 ml (distributed evenly among the 4 footpads) of complete Freund's adjuvant containing a total of 1.0 mg R-azo-BγG. Lymph nodes were removed 3–6 weeks later and microsomes were prepared as usual.

BγG (fraction II of bovine plasma) and RγG (fraction II of rabbit plasma) were obtained from Armour and Co., Chicago, Ill., and Pentex Corp., Kankakee, Ill., respectively. Twice recrystallized bovine hemoglobin and protamine sulfate were obtained from Sigma Chemical Co., St. Louis. Twice recrystallized lysozyme and crystalline papain were products of Worthington Biochemical Co., Freehold, N. J. 4,5-H^3-DL-leucine, specific activity 3,500 μcuries per μmole, was obtained from New England Nuclear Corp., Boston. I^{131} was counted in a well-type scintillation counter and H^3-labeled proteins were counted in a Packard liquid scintillation spectrometer.

Results.—Characteristics of the microsomal antibody system: In a previous report,[1] the assay for microsomal antibody activity was described with particular reference to its specificity and sensitivity. For purposes of the present work, it was necessary to characterize some other parameters of this assay. The advantage of using the hapten, ε-DNP lysine, to elute the indicator antigen after it has been bound to microsomal antibody is brought out in Table 1. If the combined radioactivity of the final supernatant and pellet for any given tube, eluted with or without hapten, is taken as the total quantity of indicator antigen bound to the microsomes, then microsomes from DNP-BγG–injected animals obviously take up far more I^{131}DNP-BSA than do the lymph-node microsomes from control animals. However, measurement of low levels of antibody activity based on the total indicator antigen bound to microsomes would be hazardous and also laborious, as it would necessitate the routine isolation of microsomes from control animals to provide an analytical blank. The requirement for the latter control is eliminated when the elution technique is used; i.e., the value for control microsomes is zero (Table 1). Since most of the indicator antigen bound to microsomes of DNP-BγG–injected animals is specifically eluted by hapten, the assay is insensitive to microsomal contamination of the final supernatant.

The selection of a 30-minute incubation period for elution (with and without hapten) is based on an experiment in which elution for 15, 30, 45, and 60 min yielded, respectively, 61, 81, 77, and 81 mμg indicator antigen specifically eluted per mg microsome protein. Elution is performed at 37°, since at 4° the release of bound indicator antigen occurs very slowly. For example, after 90 min at 4°, less than 15 per cent of the total elutable indicator antigen is released. The temperature dependency of hapten displacement of antigen from microsomes is in accord with previous observations on hapten dissociation of antibody-antigen complexes.[10]

The dependence of the assay on the amount of microsomes was shown in an experiment wherein 1.4, 2.8 and 3.7 mg microsomes protein yielded, respectively, 67, 140, and 167 mμg indicator antigen specifically eluted.

TABLE 1

EFFECT OF ELUTION ON THE DISTRIBUTION OF INDICATOR ANTIGEN BETWEEN SUPERNATANT AND MICROSOMAL PELLET

Immunized with	Eluted with	Fraction*	Radioactivity, cpm: Total	Radioactivity, cpm: Specifically eluted†
Freund's adjuvant	Medium A	Supernatant	1,100	<150
		Pellet	7,850‡	
	ε-DNP lysine§	Supernatant	975	
		Pellet	7,490‡	
DNP-BγG in Freund's adjuvant	Medium A	Supernatant	6,260	41,400
		Pellet	54,700**	
	ε-DNP lysine§	Supernatant	47,700	
		Pellet	16,000**	

The experimental procedure is described in *Materials and Methods*.

* Refers to the last step in the microsomal antibody assay in which the supernatant and microsomes (pellet) are separated by centrifugation at 105,000 × *g*.

† Difference between supernatants obtained by elution with Medium A alone and by elution with ε-DNP lysine in Medium A. 41,400 cpm corresponds to 78 mμg I^{131}-DNP-BSA eluted/mg microsomal protein.

‡ The pellet contained 2.65 mg protein.

§ 10^{-2} *M* ε-DNP lysine in Medium A.

** The pellet contained 1.95 mg protein.

For routine assay it is desirable to use an amount of the I^{131}-indicator antigen which allows saturation of the microsomes but which is not in unnecessary excess. As shown in Figure 1, the binding capacity of a representative preparation of active microsomes is estimated, by extrapolation, to be 72 mμg indicator antigen. While the amount of indicator antigen normally added (about 15 μg) does not saturate the binding capacity of this preparation, the yield of specifically eluted antigen is relatively insensitive to small variations in the quantity of indicator antigen added in the range 7–20 μg. For example, the three highest levels of antigen eluted in Figure 1, i.e., 52, 56, and 59 mμg, were obtained when 7.6, 14.2, and 17.1 μg, respectively, of indicator antigen were added.

The microsomal antibody is stable at −20° and at 4°, with a loss of less than one-third of the initial activity over a 9-day period. Ordinarily, the microsomes were stored at 4° as a suspension in medium A. Although most of the data presented herein were obtained with preparations stored up to 7 days, repetition of some of the more critical experiments (e.g., Table 5) with fresh microsomes yielded the same results.

A comparison of the antibody activity associated with the microsomal and mitochondrial fractions of a lymph node homogenate is shown in Table 2. The distribu-

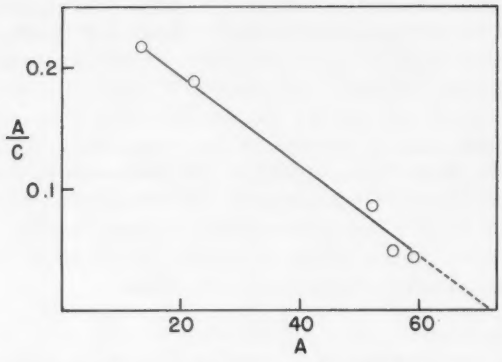

FIG. 1.—Titration of microsomal antibody activity with indicator antigen. To a constant amount of microsomes (0.85 mg protein in final pellet) varying quantities of indicator antigen were added and the microsomal antibody activity determined (see *Materials and Methods*). *A* refers to the indicator antigen specifically eluted (mμg) from the microsomes, and *C* refers to the concentration of unbound indicator antigen (mμg/ml). Total volume per tube: 12.5 ml.

tion of diphosphopyridine nucleotidase was determined in the same fractions, since this enzyme is known to be associated principally with the microsomal fraction in several rat tissues.[11] Both the enzyme and antibody activities (expressed as specific activities relative to protein) are about 4–5 times higher in microsomes (Table 2).

TABLE 2

COMPARISON OF ANTIBODY AND DIPHOSPHOPYRIDINE NUCLEOTIDASE ACTIVITIES ASSOCIATED WITH MICROSOMES AND MITOCHONDRIA

Experiment no.	Cell fraction	Antibody Activity* per mg of		Diphosphopyridine Nucleotidase† units per mg of	
		Protein	RNA	Protein	RNA
1	Mitochondria	11	390	64	1,280
	Microsomes	45	364	204	582
2	Mitochondria	10	353	50	1,008
	Microsomes	57	485	213	646

The experimental procedure for the preparation of cell fractions and the measurement of antibody activity is described in *Materials and Methods*.

* mμg antigen specifically eluted per mg protein or RNA of the cell fractions.

† Diphosphopyridine nucleotidase activity was measured in a 0.5 ml reaction volume containing 0.3 ml cell fraction in Medium A, 0.1 ml *M* Tris pH 7.6, and 0.1 ml diphosphopyridine nucleotide (6 μmoles/ml). The reaction was terminated at 15 min by the addition of 3.5 ml *M* KCN^{21}, and absorbance was determined at 325 mμ. A unit of enzyme is that amount causing the hydrolysis of 0.1 μmole of diphosphopyridine nucleotide under these conditions.

When the activities are expressed relative to RNA, antibody activity is equal in mitochondria and in microsomes, and enzyme activity is slightly higher in mitochondria than in microsomes. The mitochondria used in these experiments contained 4–5 per cent RNA, which is considerably higher than the 1–2 per cent level reported for liver mitochondria.[12] Since aggregated microsomes would tend to be recovered in the mitochondrial fraction during preparation of these cell components, the data are consistent with the view that the antibody activity is associated principally, if not exclusively, with the microsomes.

Effect of serum and some serum proteins on microsomal antibody activity: The conditions required to separate antibody activity from microsomes were most easily evaluated by measuring the loss of activity from the microsomes. In preliminary attempts to achieve the desired separation, soluble extracts of guinea pig lymph

nodes were tested on the assumption that enzymatic activity might be required. The effectiveness of several lymph-node extracts was inconstant, and the possibility that variable serum contamination of the extracts was responsible for the observed dissociation was tested with whole serum. Incubation of microsomes with normal guinea pig or rabbit serum resulted in a substantial loss of microsomal anti-DNP antibody activity (Table 3). Similar results were obtained by treating the micro-

TABLE 3

LOSS OF MICROSOMAL ANTIBODY ACTIVITY ON INCUBATION WITH NORMAL SERUM, γ-GLOBULINS, AND PURIFIED ANTIBODY

Experiment no.	Treated with	Pellet protein* (mg)	Antibody activity† (mμg/mg protein)
1	Control	0.45	46
	Normal serum‡	0.46	12
2	Control	1.56	64
	γ-globulins§	1.56	30
3	Control	0.49	44
	Antibody††	0.48	14
	Antibody**	0.50	19

Microsomes from lymph nodes of guinea pigs immunized with DNP-BγG were suspended in 5.0 ml. of medium A containing either normal serum, γ-globulins, or antibody. The controls were suspended in 5.0 ml of medium A alone. After incubation at 37° for 1 hr, the tubes were filled with medium A (12.5 ml final volume) and centrifuged for 15 min at 105,000 × g. After discarding the supernatant, the pellets were rinsed twice with medium A and then resuspended and assayed for microsomal antibody as described in *Materials and Methods*.

* The term pellet protein is used to emphasize the fact that microsomal protein, to which all values are normalized, refers to the residual pellet at the termination of the assay.

† mμg antigen specifically eluted per mg microsomal protein.

‡ 0.1 ml normal rabbit serum. Rabbit and guinea pig sera were equally effective. Microsomes treated with 1.0 ml whole guinea pig serum had exactly the same diphosphopyridine nucleotidase activity as untreated control microsomes.

§ 1.0 mg rabbit γ-globulin. Guinea pig γ-globulin was as effective as rabbit γ-globulin.

** 0.58 mg rabbit antiphenyl (*p*-azo-benzoylamino)-acetate antibody.[12]

†† 1.67 mg rabbit antiphenyl (*p*-azo-benzoylamino)-acetate antibody.[12]

somes with γ-globulin fractions of normal rabbit and guinea pig sera or with a highly purified rabbit antibody[13] whose specificity was unrelated to the DNP determinant (Table 3). The dependency of this effect on time and temperature of incubation is shown in Table 4. At 37°, the reaction is essentially complete in 15

TABLE 4

EFFECT OF TIME AND TEMPERATURE ON THE REDUCTION OF MICROSOMAL ANTIBODY ACTIVITY BY TREATMENT WITH A PURIFIED ANTIBODY

Treated with	Conditions	Pellet protein,* (mg)	Antibody activity† (mμg/mg protein)
Control	16 hr 4°	0.38	50
Antibody‡	16 hr 4°	0.38	39
Control	45 min 37°	0.35	45
Antibody‡	15 min 37°	0.40	24
"	30 min 37°	0.38	23
"	45 min 37°	0.37	22

The procedure used is the same as described in Table 3 except for the changes in time and temperature shown.

* See Table 3.

† mμg antigen specifically eluted per mg microsomal protein.

‡ 0.23 mg rabbit antiphenyl (*p*-azo benzoylamino)-acetate antibody.

min, while at 4°, the reaction is incomplete in 16 hr. In other experiments, after one hr incubation at 4° with the purified antibody of unrelated specificity there was no detectable loss of microsomal anti-DNP antibody activity.

Porter[3] has shown that papain splits rabbit γ-globulin and antibodies into three separable fractions which retain a high measure of structural integrity. Fractions I and II retain the ability to interact specifically with antigen (without precipitation), whereas fraction III is totally lacking in this activity. The availability of these

fractions made it possible to determine which segment of intact γ-globulin is responsible for reducing the level of microsomal anti-DNP antibody activity. The data of Table 5 (experiment 1) show that fraction III reproduces the behavior of

TABLE 5

EFFECT OF γ-GLOBULIN FRACTIONS ON MICROSOMAL ANTIBODY*

Experiment no.	Microsomes: From	Microsomes: Treated with	Pellet protein† (mg)	Antibody activity‡ (mμg/mg protein)
1	Guinea pig	Control	2.52	35
		I, II	2.42	36
		III	2.48	18
		I, II, III	2.57	20
2	Rabbit	Control	0.80	13
		I	0.75	10
		II	0.80	10
		III	0.75	3
3	Guinea pig	Control	1.73	30
		Hemoglobin	1.60	33
		Protamine	2.08	28, 34§
4	Guinea pig	Control	1.81	31
		Lysozyme	2.57	23, 32§

* Microsomes were prepared from lymph nodes of animals injected with DNP-BγG.
In Experiment 1, guinea pig lymph-node microsomes were suspended in 2.0 ml of Medium A which contained the indicated combinations of the fractions obtained on papain digestion of normal rabbit γ-globulin. The amounts of fractions I, II, and III were 1.0, 1.26, and 0.54 mg, respectively. The controls were suspended in 2.0 ml Medium A alone. All tubes were incubated for 1 hr at 37° and then processed as described in Table 3. The procedure followed in Experiment 2 was exactly the same as in Experiment 1, except for the use of rabbit lymph-node microsomes and the fact that the quantities of I, II, and III were 2.4, 2.9, and 0.6 mg, respectively.
Experiments 3 and 4 followed the same procedure as Experiment 1 but used 3.0 mg hemoglobin, 0.6 mg protamine, and 3.0 mg lysozyme.
† See Table 3.
‡ mμg antigen specifically eluted per mg microsomal protein.
§ The second value is corrected to the protein value of the control. Some of the added protamine and lysozyme apparently adheres to the microsomes yielding a higher pellet protein value than in the corresponding controls.

intact γ-globulin, whereas fractions I and II are ineffectual.

Following papain digestion of rabbit γ-globulin and dialysis of the reaction mixture, fraction III crystallizes as fragile plates in the shape of nonrectangular parallelograms.[3] Papain digestion of purified guinea pig antibody (anti-DNP) also resulted in the appearance of crystals which were similar to those obtained from rabbit γ-globulin except that they were rectangular plates. The solubility of fraction III of the guinea pig antibody was, however, so low (150 μg/ml 0.15 *M* NaCl—0.01 *M* phosphate pH 7.2) that this fraction could not be examined for its effect on guinea pig microsomal anti-DNP antibody activity. In order to examine the effects of papain-produced fractions of γ-globulins on microsomes of the homologous species, it was necessary, therefore, to use lymph-node microsomes from rabbits immunized with DNP-BγG. The results, shown in Table 5 (experiment 2), corroborate the observations made with guinea pig microsomes and fraction III of rabbit γ-globulin.

Specificity of γ-globulins in reducing microsomal antibody activity: Rabbit and guinea pig sera are about equally capable of reducing the antibody activity of guinea pig lymph-node microsomes. The data of Table 3 show that the effectiveness of these sera may be ascribed to their γ-globulins. Calf serum is, however, much less effective. Bovine serum albumin was entirely ineffectual, and while BγG had some slight capability, it was far less effective than RγG. With a single preparation of microsomes, the quantities of RγG and of BγG required to reduce antibody activity by 50 per cent were, respectively, 1 mg and more than 100 mg. Perhaps the strongest evidence for the specific character of the γ-globulin effect in

reducing the level of microsomal antibody is provided by the different capabilities of the three fractions obtained from rabbit γ-globulin by papain digestion (see above and Table 5).

γ-globulins have relatively high isoelectric points. Of the three fractions obtained with papain, the fraction (III) which is capable of lowering the level of microsomal antibody is relatively enriched in respect to basic amino acids.[3] The question arises, therefore, whether other basic proteins are active. As shown in Table 5 (experiments 3 and 4), hemoglobin, lysozyme, and protamine had no effect.

The specificity of the γ-globulin effect is emphasized further by the observation that the diphosphopyridine nucleotidase activity of lymph-node microsomes is completely unaffected by incubation with guinea pig serum (see legend of Table 3).

The foregoing observations suggest that the antibody activity of lymph-node microsomes may be due to antibodies which are bound through noncovalent bonds. Accordingly, active microsomes were exposed to 8.0 *M* urea (pH 7.5) and to sucrose media at low pH. Both of these conditions markedly reduced the level of microsomal anti-DNP antibody (Table 6). It may be noted in this connection that

TABLE 6

EFFECT OF UREA AND pH ON MICROSOMAL ANTIBODY

Experiment no.	Treated with	Pellet protein* (mg)	Antibody activity† (mμg/mg protein)
1	Control	0.90	76
	pH 2.0	0.79	7
	pH 3.0	0.70	10
2	Control	2.43	18
	8 *M* urea	2.38	8

In Experiment 1, microsomes from lymph nodes of guinea pigs immunized with DNP-BγG were suspended in Medium A in which TRIS was replaced by HCl in a final concentration of either 0.01 *N* (pH~2.0) or 0.005 *N* (pH~3.0). The controls were suspended in 2.0 ml Medium A alone. All tubes were incubated for 10 min in an ice bath. The tubes were then filled with the same media used for incubation (12.5 ml final volume) and centrifuged and the pellet was then assayed for microsomal antibody as described in Table 3 and *Materials and Methods.*

In Experiment 2, microsomes from lymph nodes of guinea pigs immunized with DNP-BγG were suspended in 2.0 ml of Medium A (controls) or 2.0 ml of 8.0 *M* urea in 0.01 *M* TRIS pH 7.6. After incubation for 1 hr at room temperature, the contents of the tubes were dialyzed at 4° against three successive 2-liter volumes of Medium A over an 18-hr period. Following dialysis, the microsome suspensions were assayed for antibody activity as described in *Materials and Methods.*

* See Table 3.

† mμg antigen specifically eluted per mg microsomal protein.

rabbit antibodies retain their specific reactivities despite treatment with urea at a high concentration or brief exposure to pH 2.

Microsomal antibody activity was essentially unchanged by a variety of other conditions; e.g., by cysteine (0.01 *M*) and ethylenediaminetetracetate (0.01 *M*), ATP (0.001 *M*) or pyrophosphate (0.001 *M*).

Passive cutaneous anaphylaxis reaction of microsomal antibody: Passive cutaneous anaphylaxis offers a relatively simple and sensitive assay for detecting antibody. Ordinarily, this assay is performed by the intradermal injection of antibody followed several hours later by intravenous injection of the homologous antigen mixed with a dye. Specific interaction of antigen and antibody in the skin results in local increase in capillary permeability and extravasation of dye at the skin site.[14]

Skin sites that had been injected with a microsome suspension obtained from DNP-BγG–immunized animals reacted to intravenous injection of DNP-HSA, whereas sites that had been injected with 18 times more microsomes from control animals (immunized with benzene arsonate azo-protein) failed to react (Table 7). The positive skin reactions were completely inhibited, in other experiments, by

TABLE 7

PASSIVE CUTANEOUS ANAPHYLAXIS PRODUCED WITH A SUSPENSION OF MICROSOMES

Microsomes							
From animals immunized with	Amount injected, mg protein	Diameter (mm) of response for animal number					
		1	2	3	4	5	6
R-azo-BγG*	0.218	0	0	0	—	—	—
DNP-BγG	0.012	0	5	5	15	12	10

Guinea pigs (~250 gm) were injected intradermally on their ventral surface with 0.1 ml of microsome suspension in Medium A. Five hr later, the animals were injected intravenously with 0.5 ml containing 1 mg DNP-HSA and 2.5 mg of a blue dye (Evan's blue). Fifteen min later, the animals were sacrificed and diameters of extravasated blue dye at sites of intradermal injection were measured. For additional details of this assay, see text and reference 14. The microsomes injected had the usual protein/RNA weight ratio of ~3.0.[2, 16]

* *p*-arsonic acid benzene azo-bovine γ-globulin.

intraperitoneal injection of hapten (35 μmoles ε-DNP lysine) 30 min prior to challenge with the antigen-dye mixture. Hapten inhibition of the skin test established its specificity for the DNP group and confirms the recent findings of Ovary and Karush in respect to the susceptibility of this skin response to hapten inhibition.[15]

Although in these experiments the skin sites were injected with microsome suspensions, it is not known whether anti-DNP antibody activity was still associated with microsomes at the time of challenge with antigen. Indeed, in view of the results of Table 3, it is likely that the antibody activity was separated from the microsomes in the skin sites by the γ-globulins of the test animals.

The smallest quantity of antibody detectable by passive cutaneous anaphylaxis has been reported to be about 0.01 μg.[14] If this sensitivity is assumed for the experiments in Table 7, the microsome preparation used had about 1 μg antibody per mg protein.

Demonstration of soluble antibody released from microsomes: The assay for microsomal antibody can only be used to measure antibody activity associated with sedimentable particles. Other procedures were required to determine whether soluble antibody was released from the microsomes by those conditions which reduced the level of microsomal antibody activity (Tables 3–6). As shown in Table 8, microsomes exposed briefly to pH 2–3 yielded soluble antibody which was detect-

TABLE 8

PASSIVE CUTANEOUS ANAPHYLAXIS PRODUCED WITH ANTIBODY RELEASED FROM MICROSOMES

Microsomes treated with	Microsome supernatant dilution	Diameter (mm) of response for animal number	
		1	2
Control	1:3	0	3*
pH 2.5	1:3	10	15
Control	1:6	—	0
pH 2.5	1:6	—	5

Microsomes from guinea pigs immunized with DNP-BγG were suspended in 2.0 ml unbuffered Medium A (controls) or Medium A in which TRIS was replaced by HCl at a final concentration of 0.01 *N* (pH 2.5). After 10 min in an ice bath, both samples were adjusted to pH 5.2 with 0.4 *M* acetate buffer and centrifuged for 10 min at 9,000 × *g*. The supernatant was neutralized with NaOH to about pH 7.2 and diluted in Medium A. Dilutions of the supernatant were injected intradermally and passive cutaneous anaphylaxis was elicited and the response measured as described in Table 7.

* Faint color.

able by passive cutaneous anaphylaxis.[16]

An alternate and independent method for detecting the release of soluble antibody from microsomes involved the use of a particulate fraction derived from cells which had incorporated H^3-leucine of high specific activity into anti-DNP antibody. Using procedures described by Helmreich *et al.*,[8] isolated lymph-node cells from DNP-BγG–immunized guinea pigs were incubated with H^3-leucine and then

homogenized, and a particulate fraction was isolated by differential centrifugation (Table 9). After exposing the particulate fraction (mitochondria and microsomes) briefly to pH 2.5 at 4°, the particles were sedimented and the supernatants were assayed for H^3-labeled anti-DNP antibody by coprecipitation with carrier antigen and antibody.[8] The results, given in Table 9, demonstrate clearly the dissociation

TABLE 9

COPRECIPITATION OF ANTIBODY RELEASED FROM PARTICULATE FRACTION OF CELLS

Experiment no.	Particles treated with	Radioactivity in anti-DNP antibody,* cpm
1	Control	79
	pH 2.5	1,119
2	Control	109
	pH 2.5	3,480
3	Control	221
	pH 2.5	7,330

8–10 reaction mixtures consisting each of 2–3 × 10^8 guinea pig lymph-node cells in 2.0 ml of medium containing 88 μc H^3-DL-leucine were incubated at 37° for 1 hr under 95 per cent O_2–5 per cent CO_2. The cells were harvested by centrifugation (1,300 × g), lysed with a small volume of H_2O, and homogenized in Medium A. The homogenate was centrifuged at 2,500 × g for 10 min and the sediment discarded. The supernatant was divided in half and centrifuged in separate tubes at 105,000 × g for 1 hr. One of the pellets was suspended in unbuffered Medium A (control) and the other in Medium A in which TRIS was replaced by HCl at a final concentration of 0.01 N (pH 2.5). After 10 min in an ice bath, both samples were adjusted to pH 5.2 with 0.4 M acetate buffer and centrifuged for 10 min at 9,000 × g, and the supernatant was neutralized with NaOH to about pH 7.2.

In Experiments 1 and 2, 0.58 mg DNP-BγG and 3.5 mg anti-DNP antibody (in the form of a γ-globulin fraction) were added to the neutralized supernatant yielding a specific precipitate. In Experiment 3, the neutralized supernatant was subjected to further centrifugation for 4 hr at 93,000 × g before addition of the carrier antigen and antibody.

* Specifically eluted with ϵ-DNP lysine from the specific precipitate made with DNP-BγG and anti-DNP antibody.[8]

at low pH of soluble H^3-antibody from the particles.

Discussion.—The foregoing observations indicate that microsomal antibody activity is a function of microsome-antibody complexes; i.e., conventional antibody (γ-globulin) linked to microsome particles. The principal evidence in support of this interpretation is the fact that brief treatment of an active microsome preparation at low pH leads to a decrease in antibody activity of the particles with simultaneous appearance in the soluble phase of material which exhibits the behavior expected of conventional antibody, i.e., reactivity in passive cutaneous anaphylaxis and coprecipitation with homologous carrier antibody and antigen. This interpretation is further supported by the restricted nature of the proteins which are capable of reducing the level of microsomal antibody activity. Of the several proteins examined, from serum and other sources, only γ-globulins dissociated the complex. Rabbit and guinea pig γ-globulins were particularly effective, but bovine γ-globulin had hardly any effect. Moreover, of the fractions resulting from papain digestion of rabbit antibody or γ-globulin, only crystalline fraction III reduced the level of antibody activity on microsomes. These observations emphasize the specificity of the antibody-microsome interaction and imply that structural complementarity between microsomal sites and antibody (γ-globulin) is involved in this interaction.

The dissociation of the microsome-antibody complex by urea or by brief exposure to pH 2.5 and the specific dissociation of these complexes by certain γ-globulins, including purified rabbit antibodies and crystalline fraction III, suggest that the linkages involved in establishing the complex are not covalent.

In view of the structural heterogeneity of the microsome fraction, the question arises whether antibodies found in this fraction are bound to the microsomes *per se*

(i.e., ribosomes) or to other components of the endoplasmic reticulum.[17] At present, it is not possible to answer this question, since a procedure which selectively separates the ribosomes from the endoplasmic reticulum without otherwise altering these components is not available. Although deoxycholate has been used to prepare ribosomes from mammalian tissues,[17] this reagent extracts considerable amounts of protein from the microsomal fraction,[18] and it also solubilizes or inactivates most of the microsomal antibody activity. In a representative experiment, when lymph-node microsomes[2] were treated with 0.25 per cent deoxycholate and then centrifuged at 12,000 $\times$ g for 15 min, an opaque white sediment was obtained (weight ratio protein/RNA = 6.3); when the supernatant was then recentrifuged at 100,000 $\times$ g for one hr, the transparent yellow pellet obtained was judged to be ribosomes on the basis of its having a protein/RNA weight ratio of 1.1. The low-speed RNA-poor sediment and the ribosomal pellet each had only 5 per cent of the antibody activity of the original microsomal fraction from which they had been derived. When the latter antibody activities are expressed as mμg indicator antigen specifically eluted per mg protein, or per mg RNA, or per mg protein + RNA, the residual antibody activity of the ribosomes was 12, 17, and 7, respectively, as compared to 69, 234, and 53, respectively, for the original microsomal fraction. Whether the 90 per cent of the microsomal antibody activity not accounted for after deoxycholate treatment was inactivated or solubilized was not determined. Although currently favored views might suggest that microsomal antibodies should be associated with ribosomes, we cannot exclude the possibility that these antibodies are, at least in part, associated with microsomal structures which in the cell constitute the endoplasmic reticulum.[19]

While there is no direct evidence that the antibodies associated with microsomes are synthesized on these particles, it may be noted that in cell-free lymph-node extracts, which incorporate isotopically labeled amino acids into protein, treatment of the microsomal fraction with a detergent yields soluble isotopically labeled protein that is immunologically indistinguishable from γ-globulin.[20]

The present findings and their interpretation furnish the following tentative explanation for the assay used for detecting microsomal antibody activity. We visualize that components of the microsomal fraction bind reversibly that portion of an antibody molecule (or γ-globulin) which corresponds to fraction III of a papain digest. The residual segments of the antibody molecule (fractions I and II) bear the two active sites which are then accessible for interaction with the indicator antigen. Since it is further visualized that the specific dissociation of the microsome-antibody complex by crystalline fraction III involves an exchange, it is expected that lymph-node microsomes from an unimmunized guinea pig should bind antibodies added to them *in vitro*. Binding of this type was described in a previous communication.[1]

Experiments concerned with identifying the particular microsomal constituent which specifically interacts with the fraction III moiety of rabbit and guinea pig antibodies are in progress.

Summary.—When lymph-node microsomes are treated under a variety of conditions, their antibody activity is diminished and antibodies may be identified in the soluble phase. The conditions which are effective in separating antibodies from microsomes indicate that the linkages involved are not covalent. It is inferred that

the microsome-antibody complex is reversible and due to a specific interaction between microsomal components and that segment of antibody (or γ-globulin) which corresponds to the crystallizable fraction obtained by papain digestion of rabbit γ-globulin.

We are grateful to Mr. John Courtney for valuable technical assistance.

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[1] Kern, M., E. Helmreich, and H. N. Eisen, these PROCEEDINGS, **45**, 862 (1959).

[2] The term microsome refers to that fraction of a lymph-node homogenate which is not sedimentable at 15,000 × *g* (15 min) but is sedimentable at 105,000 × *g* (1 hr). The weight ratio of protein/RNA in guinea pig lymph node microsomes is ordinarily about 3.2–3.5. At the termination of assay for microsomal antibody, this ratio is about 8.0–8.8. In ribosomes prepared by deoxycholate treatment of microsomes (see discussion), the protein has an $E^{1\%}_{1cm}$ value of 7.0 (280 mμ, 5.2 *M* acetic acid).

[3] Porter, R. R., *Biochem. J.*, **73**, 119 (1959).

[4] Abbreviations used: DNP is used generically to indicate 2,4-dinitrophenyl groups combined with proteins or amino acids; DNP-BγG is used for 2,4-dinitrophenyl bovine γ-globulin; DNP-BSA for 2,4-dinitrophenyl bovine serum albumin; DNP-HSA for 2,4-dinitrophenyl human serum albumin: ε-DNP lysine for ε-2,4-dinitrophenyl lysine; BγG for bovine γ-globulin; RγG for rabbit γ-globulin; Razo BγG for *p*-arsonic acid benzene azo-bovine γ-globulin; TRIS for tris (hydroxymethyl) aminomethane.

[5] Littlefield, J. W., and E. B. Keller, *J. Biol. Chem.*, **224**, 13 (1957).

[6] Schneider, W. C., *J. Biol. Chem.*, **164**, 747 (1946).

[7] Schneider, W. C., in *Manometric Techniques*, eds. W. W. Umbreit, R. H. Burris, and J. F. Stauffer (Minneapolis: Burgess Publishing Co., 1947), p. 194.

[8] Helmreich, E., M. Kern, and H. N. Eisen, *J. Biol. Chem.*, **236**, 464 (1961).

[9] We wish to thank Dr. C. W. Parker for preparing this reagent.

[10] Farah, F. S., M. Kern, and H. N. Eisen, *J. Exp. Med.*, **112**, 1195 (1960).

[11] Jacobson, B. K., and N. O. Kaplan, *J. Biophys. and Biochem. Cytol.*, **3**, 31 (1957).

[12] Greengard, O., *Biochim. et Biophys. Acta*, **32**, 270 (1959).

[13] We are indebted to Dr. F. Karush of the University of Pennsylvania for his generous gift of purified antiphenyl (*p*-azo-benzoylamino)-acetate antibody.

[14] Ovary, Z., *Progress in Allergy*, **5**, 459 (1958).

[15] Ovary, Z., and F. Karush, *J. Immunol.*, **84**, 409 (1960).

[16] In another experiment which followed the protocol of Table 8, the final microsomal pellets for the pH 2.5–treated sample and the control sample contained, respectively, 1.56 and 1.51 mg protein. This should be compared to a value of 1.5 mg protein for an equal aliquot of microsomes which was untreated (directly precipitated with trichloroacetic acid).

[17] Palade, G. E., in *Microsomal Particles and Protein Synthesis*, ed. R. B. Roberts (New York: Pergamon Press, 1958), p. 36.

[18] Peters has shown that deoxycholate extracts the chicken serum albumin that is bound to the microsomal fraction of chicken liver, *J. Biol. Chem.*, **229**, 659 (1957).

[19] The widespread view that ribosomes exercise a specificity-determining function in protein synthesis implies that ribosomal populations are heterogeneous and differentiated. If this is true, and if the antibody attached to microsomes is attached to ribosomes and is related to the synthetic process, it would be expected that only a small fraction of the lymph-node ribosomes are associated with antibody. This possibility may be tentatively evaluated from estimates of the amount of antibody found in lymph-node microsomes. From titration with I^{131} DNP-BSA (Fig. 1), this amount appears to be about 0.1 μg antibody/mg protein. This value is probably more valid than that estimated from passive cutaneous anaphylaxis (1 μg/mg protein), but actually both values are in fair agreement when normalized to RNA (1–3 μg antibody/mg RNA; see footnote 2 and

legend of Table 7). If it is assumed that the antibody is attached only to ribosomes and that all of the RNA of the microsomal fraction is in ribosomes, it may be estimated that there is about 1 antibody molecule per 100–200 ribosomal particles. Or, if about 10 per cent of the cells in the lymph nodes taken for homogenization contain microsomal anti-DNP antibody, it appears that in such cells only one out of about 10–20 ribosomes bears an antibody molecule. On steric grounds, it is unlikely that a single antibody molecule could simultaneously bind more than 1 or 2 ribosomes, but a single ribosome might carry many more than 1 antibody molecule. While these numerical speculations are consistent with the possibility of ribosomal differentiation, their acceptability is seriously limited by uncertainty as to what fraction of the total γ-globulin produced by an antibody-synthesizing cell is antibody of a given specificity.

[20] Eisen, H. N., E. Simms, E. Helmreich, and M. Kern, unpublished results.

[21] Colowick, S. P., N. O. Kaplan, and M. M. Ciotti, *J. Biol. Chem.*, **191**, 473 (1951).

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*ULTRAVIOLET LIGHT INDUCED LINKING OF DEOXYRIBONUCLEIC ACID STRANDS AND ITS REVERSAL BY PHOTOREACTIVATING ENZYME**

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The effects of ultraviolet light (UV) on compounds of biological interest have been the subject of numerous investigations. In a recent excellent and comprehensive review, Shugar[1] has described and evaluated the effects of UV on nucleic acids and its constituents. At the *chemical level* it would appear that of the deoxyribonucleic acid (DNA) bases, the pyrimidines are altered more readily than the purines.[2] Recent work[3] pertaining to the photochemistry of nucleic acids has opened a new dimension in the field by invoking thymine dimer formation when this pyrimidine is irradiated with UV either in the frozen state or when in its more natural habitat, DNA (in solution).[4–6] At the *physical chemical* level of UV induced DNA damage, many changes have been detected and described, but the most sensitive thus far would appear to be the lowering of the thermal denaturation temperature of native DNA.[7, 8] At the *biological level*, the inactivation of transforming factor DNA, while not as sensitive as the killing of bacteriophage, offers the most readily approachable means of studying the end result of the UV irradiation on DNA at the macromolecular level. The purpose of the present communication is to present some new data bearing on the effects of UV on DNA at the macromolecular level, using physical chemical, biological, and enzymatic tools, with the hope that it may be useful in explaining the effects that occur *in vivo*.

Materials and Methods.—Transformation: Transformation of *Diplococcus pneumoniae* (R-36A) was carried out according to the method of Fox and Hotchkiss.[9] *Bacillus subtilis* (168) transformation was carried out as described by Spizizen.[10]

Deoxyribonucleic acid: DNA was isolated from exponentially grown cells by the method of Marmur.[11] Hybrid N^{14}–N^{15} *Escherichia coli* B DNA was isolated from cells first grown for many generations in $N^{15}H_4Cl$ as the sole nitrogen source followed by one generation (doubling of the turbidity) in $N^{14}H_4Cl$.[12] P^{32}-labeled DNA was isolated from *B. subtilis* (WT $E_rA_rB_r$, resistant to the antibiotics erythromycin, amycetin, and bryamycin) grown on a medium containing the following ingredients: 1 mg $MgSO_4$, 0.1 mg $MnSO_4 \cdot 7H_2O$, 0.05 mg $FeSO_4 \cdot 7H_2O$, 500 mg NH_4Cl, 100 mg

NH_4NO_3, 100 mg Na_2SO_4, 100 cc 1 *M* Tris buffer, pH 7.4, 0.2% glucose, and 5 g of Bactopeptone (which contains approximately 0.2% phosphate) and 15 mc of P^{32} in a total volume of 1,000 ml. The purified DNA had a specific activity of 1.5×10^6 cpm per mg.

Other DNA samples used in this study were isolated from *Streptomyces viridochromogenes* (94), *D. pneumoniae*, *Pseudomonas aeruginosa* (B-23), and *Clostridium perfringens* (87*b*).

CsCl density-gradient centrifugation: The method employed is similar to that described by Meselson, Stahl, and Vinograd.[13] DNA in approximately 5.7 *M* CsCl was centrifuged at 44,770 rpm for approximately 24 hr and photographed using ultraviolet optics. By using a standard DNA of known density, the buoyant densities of the banded DNA were calculated after first tracing the photographs with a Joyce-Loebl microdensitometer.

Ultraviolet irradiation: DNA solutions were irradiated at a distance of 5 cm using a G15T8 Westinghouse sterilamp. At this distance the light intensity was calculated to be 176×10^4 ergs per min per ml[2] (calculated using the rate of decrease of optical density of 1,3-dimethyluracil and its quantum yield (Wang, personal communication)). DNA solutions were irradiated 1–1.5 mm in depth in open vessels. Under these conditions, 1.2 min irradiation corresponds to 90 per cent inactivation of the streptomycin transforming activity of *D. pneumoniae* and 6–6.2 min to 99% inactivation.

Formamide denaturations: To denature DNA prior to CsCl density-gradient centrifugation, samples were dialyzed against two changes (one hour each, shaking at 37°C) of 100 volumes of 96–98% formamide (Fischer Reagent Chemical) containing 0.015 *M* NaCl plus 0.0015 *M* sodium citrate at neutral pH.[14] The formamide was removed by dialysis prior to centrifugation.

Complement fixation: Lupus erythematosus serum (L. E. serum) was obtained from J. W., a patient at the Lemuel Shattuck Hospital, Boston. The sera have previously been shown[15] to contain antibodies to denatured DNA. Quantitative complement fixation experiments were carried out by a micro technique described by Wasserman and Levine.[16]

Phosphodiesterase assay: E. coli B phosphodiesterase was prepared according to the method of Lehman who showed it to act on denatured but not on native DNA.[17] The progress of the enzymatic reaction was followed by the release of P^{32}-labeled deoxyribonucleotides from P^{32}-labeled *B. subtilis* DNA heated under denaturing conditions. The reaction mixtures contained 14.7 μg heat denatured P^{32}-labeled *B. subtilis* DNA, 2 μmoles $MgCl_2$, 20 μmoles glycine buffer, pH 9.2, 1 μmole of 2-mercaptoethanol, and 15 units of enzyme in a total volume of 0.3 ml. Incubations were carried out at 40°C and the reactions stopped by the addition of 0.2 ml of normal calf thymus DNA (2.5 mg per ml) and 0.5 ml of 3.5% cold perchloric acid. The reaction mixture remained at 0°C for 5 min and was then centrifuged at $10,000 \times g$ for 15 min. An aliquot (0.2 ml) was plated on an aluminum planchet and 1 drop each of 1 *M* KOH and concentrated sodium lauryl sulfate were added.

Photoreactivation: Photoreactivating enzyme from bakers' yeast was generously supplied by C. Rupert and R. M. Herriott. A preparation isolated by M. L. Coval and F. Bergmann in this laboratory, using the same initial fractionation as that described by Rupert[18] as well as DEAE adsorption and elution was also used in some experiments. Photoreactivation was carried out at 37°C in a thermostatted water bath at a distance of 2 cm from a Sylvania 15 watt "cool white" (F15T8-CW) lamp. The reaction was terminated by immersing the sample in an ice bath.

Experimental.—From the quantum yield of the UV induced photolysis of the DNA bases, it would appear that an early step in the photochemical damage of the DNA macromolecule is the alteration of some of the cytosine residues.[1, 2] These events, distributed along the DNA molecule would likely result in a melting[2] of some of the hydrogen bonds in the area of the lesion, resulting in a weakening of the double stranded structure. Such a UV induced sequence of events would satisfactorily explain the observations of the lowering of the thermal denaturation temperature,[7, 8] the increase in extinction coefficient at relatively low doses, the broadening of the transition range and the elevation of the pH_s of the acid induced helix to coil conversion of DNA and, in part, the increased buoyant density in the CsCl gradient.[8]

It has been shown by Levine *et al.*[19] and Stollar and Levine[15] that the heat-de-

natured species of DNA reacts preferentially with homologous antibodies as well as those obtained from the sera of patients with lupus erythematosus. Native DNA, which has been UV irradiated, however, shows a progressive increase in complement fixing ability without prior heat denaturation (Fig. 1). The implica-

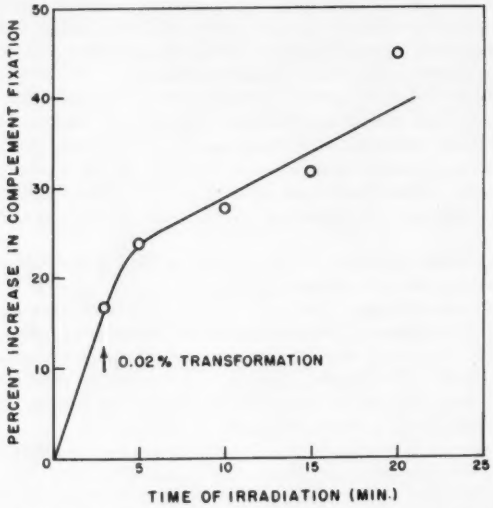

FIG. 1.—Effect of ultraviolet light on the ability of *B. subtilis* DNA to fix complement. Samples, removed after various UV doses, were assayed for complement fixation using lupus erythematosus serum. The ordinate represents the percentage complement fixed and is a relative value. It neither quantitates the extent of hydrogen bonds broken nor relates the maximum breakage in heat denatured DNA. The arrow represents the dose corresponding to 0.02% remaining transforming activity with respect to the indole marker.

tion of these data is that one of the effects of UV light is to cause breakage of hydrogen bonds exposing antigenically reactive groups. These findings were corroborated when it was also found that the UV irradiated DNA had a higher extinction coefficient and reacted with formaldehyde; both criteria are suggestive of denaturation.[20, 21] It has also been shown by utilizing these experimental techniques, as well as bacterial transformation, that under conditions of renaturation[22] reformation of hydrogen bonds broken by UV did not occur. An attempt to locate these UV induced nonhydrogen bonded areas has been made using the *E. coli* phosphodiesterase and P^{32}-labeled *B. subtilis* DNA. When the DNA was irradiated, it remained completely refractory to this enzyme which is known to require one free end of strand-separated DNA molecules in order to digest them. Thus, if one assumes that the enzyme is capable of acting, even in part on the denatured regions, the UV induced nonhydrogen bonded areas would appear to be internal rather than at the ends of the molecule.[23] The localized melting of the hydrogen bonds might result from UV induced photochemical alterations of the pyrimidine residues.

The recent experiments on dimer formation[3–6, 24] led us to think that UV might also cause other changes in the DNA molecule which would account, in part, for the

loss of biological activity. Two methods which proved useful in studying this possibility were the CsCl density-gradient technique and the use of specific enzymatic hydrolysis. The *E. coli* phosphodiesterase described by Lehman[17] hydrolyzes heat denatured and ϕX 174 DNA exonucleolytically releasing deoxynucleoside-5′-phosphates.

The possibility was first investigated whether some form of cross-linking (exochain) could be detected between homologous double stranded DNA molecules. Setlow and Doyle[25, 26] and Shugar and Baranowska[27] have shown that the irradiation of films of DNA resulted in the formation of insoluble fibers with a high quantum yield. Thus, a mixture of *S. marcescens* DNA labeled with N^{15} and deuterium[28] and normal DNA from the same source was UV treated at concentrations of 200 μg per ml each in 0.15 *M* NaCl plus 0.015 *M* Na citrate. Various doses of UV light were employed in excess of those inactivating the *D. pneumoniae* streptomycin marker to levels less than 1.0 and 0.1 per cent remaining activity. The irradiated DNA was centrifuged in a CsCl gradient. Since material banding at intermediate densities was not detected, it can be concluded that no exochain cross linking was caused by irradiating DNA in solution within the range of biological doses.

The possibility still existed that heat or formamide denaturation stable linkages could be induced *between* the strands (interchain) of the same DNA molecule or *within* a single strand (intrachain). Linkages between certain bases might occur following the irradiation induced formation of localized denatured lesions along the DNA molecule. Such distortions would provide more favorable steric conditions and enhance the interaction between base residue pairs during the irradiation. The formation of such cross-links holding the two strands together when exposed to denaturation conditions which would normally cause their separation was tested using the density-gradient technique. Hybrid N^{14}-N^{15} *E. coli* DNA (20 μg per ml) was irradiated in the native state at an ultraviolet dose sufficient to reduce streptomycin transforming activity of *D. pneumoniae* DNA to 1–1.5 per cent. UV treated, formamide denatured, and nondenatured samples were then centrifuged in a CsCl gradient. The tracings of the ultraviolet absorption photographs are shown in Figure 2 where it can be seen that UV irradiation alone increases the density of the hybrid *E. coli* DNA from 1.717 to 1.722 gm per cc. It can also be seen in the upper tracing of Figure 2 that the irradiation does not result in strand separation. In other experiments where higher doses were employed, the buoyant density of the irradiated DNA was higher. The increase might be due to the photochemical alteration of the cytosine residues and/or reflect the formation of nonhydrogen bonded regions. At the higher doses, again no strand separation was observed by the UV irradiation alone. The buoyant density increase is also related to the base composition of the DNA; the higher the adenine plus thymine content, the greater the absolute increase in density.[8] When, however, the irradiated DNA was denatured with formamide,[14] three bands were obtained instead of the two formed with nonirradiated hybrid DNA (Fig. 2). Two of the bands can be accounted for by single stranded N^{14} and N^{15} containing DNA; these correspond to the buoyant densities of 1.729 and 1.745 gm per cc. These densities are higher than those of the single-strand, nonirradiated species, and may be attributable to the alteration of the cytosine residues. The appearance of the third *E. coli* band in the lower tracing (1.723 gm per cc) can best be explained as a new molecular species in

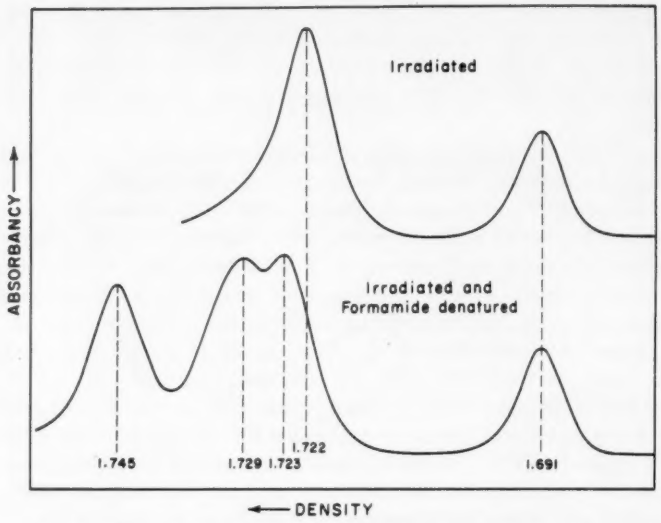

FIG. 2.—Effect of ultraviolet light and denaturation on the banding properties of hybrid $N^{14}N^{15}$ *E. coli* B DNA in a CsCl density gradient. Irradiated and denatured samples (together with a standard *C. perfringens* DNA sample (density = 1.691 gm per cc)) were centrifuged at 44,770 rpm for approximately 24 hr, the resulting bands photographed using UV absorption optics and then traced. The upper microdensitometer tracing is that of hybrid $N^{14}N^{15}$ *E. coli* B DNA banded in the CsCl gradient after first being irradiated for 6 min at a concentration of 20 μg per ml in 0.15 *M* NaCl plus 0.015 *M* sodium citrate. The bottom tracing is an aliquot of the same irradiated material banded after denaturation in formamide.

which the two complementary Watson-Crick strands are held together by cross-links which cannot be disrupted by formamide treatment. Experiment using thermal denaturation[22] instead of formamide treatment yielded similar results. However, the formamide denaturation following the UV irradiation is to be preferred since it causes little or no backbone breakage or depurination and thus increases the resolution of the CsCl density technique of detecting the cross-linked DNA molecules.

Another series of experiments was undertaken in an attempt to correlate the loss of transforming activity of *D. pneumoniae* DNA with the extent of UV induced, formamide-resistant molecules. The DNA was irradiated at doses within the biological range and the transforming activity and formamide-resistant portion measured using the CsCl technique. Formamide treatment alone would be expected to give rise to a single band of denatured DNA with a buoyant density of 0.015–0.016 gm per cc higher than that for native DNA. This is seen to be the case in the upper tracing in Figure 3. When the DNA is irradiated, however, at several doses, increasingly larger portions of the DNA become resistant to denaturation. This can be seen from the areas under the tracings of the two *D. pneumoniae* bands. After 6 min of irradiation, which inactivates the specific transforming activity with respect to the *D. pneumoniae* streptomycin resistance marker to 1–1.5 per cent of its original activity, approximately 25–30 per cent of the DNA remains resistant to

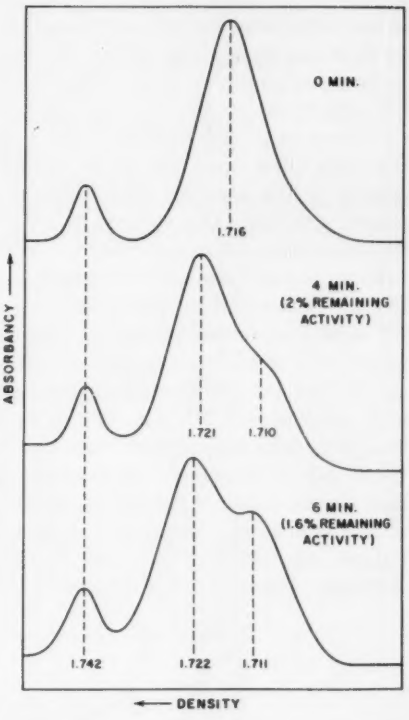

FIG. 3.—Effect of ultraviolet light and denaturation on the banding properties of *D. pneumoniae* DNA in a CsCl density gradient. Irradiated and denatured samples (together with a standard N^{15} *P. aeruginosa* samples (density = 1.742 gm per cc)) were centrifuged at 44,770 rpm for approximately 24 hr, the resulting bands photographed using UV absorption optics and then traced. The upper microdensitometer tracing represents unirradiated, formamide denatured *D. pneumoniae* DNA. The center tracing represents DNA irradiated in the native state for 4 min at 20 μg per ml in 0.15 *M* NaCl plus 0.015 *M* Na citrate (2 per cent remaining streptomycin transforming activity), denatured in formamide and banded in a CsCl gradient. In the bottom tracing the dose was increased to 6 min (1.6 per cent remaining streptomycin transforming activity), formamide denatured and then banded in a CsCl gradient.

formamide denaturation. The remaining material, which is denaturable by the formamide treatment, might of course be biologically inactive because of *intra*chain links and/or irradiation induced lesions which precede the formation of the interchain cross-links, etc. Preliminary experiments in which single stranded *D. pneumoniae* DNA was irradiated with UV light have indicated that some lesions (eliminated by the photoreactivating enzyme) are formed which resist, in part, the action of *E. coli* phosphodiesterase; the resistant material can be detected by CsCl density-gradient centrifugation.

Two pertinent preliminary observations should be mentioned at this time. First, the extent of cross-linking between strands depends on the base composition of the irradiated DNA. Thus, we have found that DNA isolated from *Streptomyces viridochromogenes* which possesses approximately 26 per cent adenine plus thymine, yields less cross-linked, denaturation resistant material for the same UV doses as does DNA from *D. pneumoniae* with an adenine plus thymine content of 62 per cent.[29] Second, when DNA was isolated from cells of *B. subtilis* subjected to biological UV doses, it contained molecules which were resistant to formamide denaturation and behaved on CsCl density-gradient centrifugation in a similar manner to *in vitro* irradiated DNA.

Another method which has proved to be very useful in detecting irradiation induced linking in the DNA molecules is the use of the phosphodiesterase from *E. coli*.[17] This enzyme initiates sequential hydrolysis from the 3′-hydroxyl end only

of heat denatured and ϕX DNA, and as such provides an extremely sensitive tool for detecting that portion of the UV treated DNA moleçules which are susceptible to heat denaturation. Heat denatured P^{32}-labeled *B. subtilis* DNA, under the heat denatured samples were then assayed for release of P^{32} nucleotides following conditions employed, quantitatively released labeled deoxyribonucleotides whereas this same DNA when exposed to UV light and then heat denatured yielded only a portion of the expected deoxyribonucleotides on enzymatic hydrolysis. It appeared then that DNA exposed to UV irradiation becomes less susceptible to heat denaturation and thus more resistant to the action of the *E. coli* phosphodiesterase.

Experiments were now performed to ascertain whether UV irradiation of P^{32} DNA was repaired by photoreactivation. *B. subtilis* P^{32} DNA was exposed to UV light for varying periods of time and incubated, under visible illumination, either with or without the photoreactivating (PR) enzyme.[18] The samples were then boiled for 10 min and immediately chilled. The heating period served to form single-strand DNA as well as to terminate the PR enzyme activity. The heat denatured samples were then assayed for release of P^{32} nucleotides following the *E. coli* phosphodiesterase hydrolysis. It can be seen from these data (Fig. 4) that the inhibition of labeled nucleotide release was directly related to the extent of UV irradiation. Incubation of these DNA samples with the PR enzyme from bakers' yeast resulted in partial recovery from the damage caused by UV light. Exposure of the irradiated P^{32}-labeled DNA to pancreatic DNase, an endonuclease,

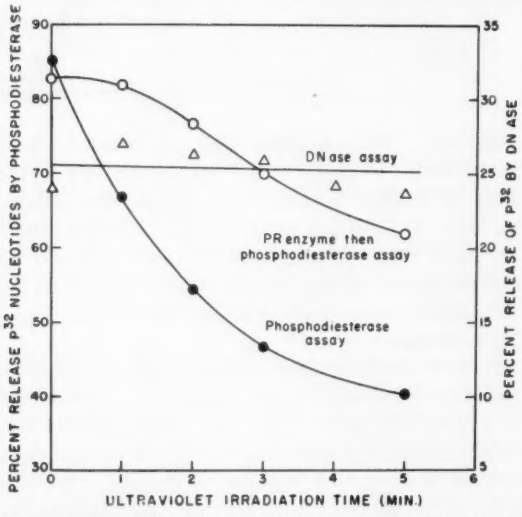

FIG. 4.—Effect of ultraviolet light and photoreactivating enzyme on P^{32}-labeled *B. subtilis* DNA. The labeled DNA was exposed to various doses and incubated, with visible illumination, either with or without the photoreactivating enzyme (1 mg per ml). Each sample was denatured for 10 min at 100°C. and immediately chilled. The denatured DNA samples were then assayed for release of P^{32} nucleotides following phosphodiesterase hydrolysis. Samples were also exposed to pancreatic deoxyribonuclease and their hydrolysis assayed by release of P^{32} (ordinate at right). Crystalline pancreatic DNase (0.1 μg per ml) was added to the P^{32}-labeled DNA as well as 3 μmoles Mg^{++}, 20 μmoles potassium phosphate buffer, pH 6.85 in a total volume of 2.0 ml. Carrier DNA and perchloric acid were added to terminate the reaction as described in the section on methods.

resulted in no significant change in the substrate susceptibility to this enzyme which acts on native as well as denatured DNA.

In order to determine whether light was required for photorecovery as well as the optimum incubation conditions for such repair, P^{32}-labeled *B. subtilis* DNA was exposed to UV light for 1 and 3 min followed by incubation at 37°C with the PR enzyme for varying time periods. Control samples containing the PR enzyme were shielded from visible light during the incubation period. The incubated samples were then heat denatured and assayed for release of labeled deoxyribonucleotides by the phosphodiesterase. It can be seen (Fig. 5) that there is a strict

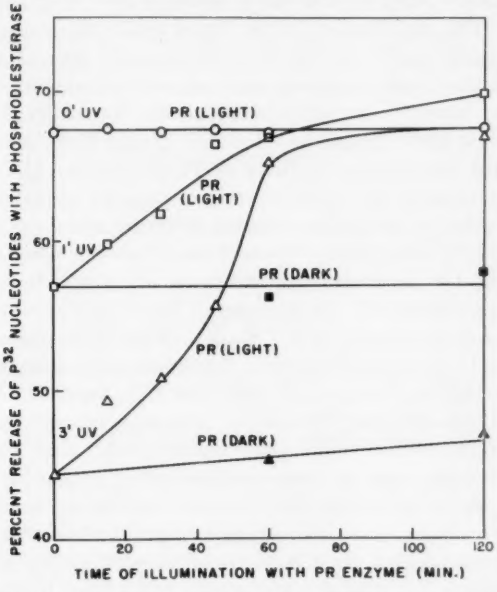

FIG. 5.—Effect of ultraviolet light and photoreactivating enzyme on P^{32}-labeled *B. subtilis* DNA. DNA was exposed to UV light for 0, 1 and 3 min followed by incubation at 37°C with photoreactivating enzyme for varying periods of time up to 120 min. Control samples were shielded from light during the incubation period. The incubated samples were thermally denatured (10 min at 100°C and immediately chilled) and assayed for release of P^{32} nucleotides following phosphodiesterase hydrolysis.

requirement for visible illumination to repair the damage imposed by UV light and that heat resistant linkages on the same or opposite strands could account for the resistance of UV irradiated DNA to exonucleolytic hydrolysis.

Discussion.—Evidence has been presented that the effects of UV light at the macromolecular level leads to partial denaturation of the DNA molecules as well as to the formation of heat and formamide denaturation resistant cross-links. The demonstration that the irradiation of thymine leads to the formation of *stable* dimers[3-6, 24] provides some precedent for the UV induced formation of the cross-links in DNA between thymine residues. Isolation of thymine dimers from UV irradiated DNA[4-6] following degradation has recently been reported. Cross-linking of the DNA strands, which also occurs when whole cells are irradiated with

UV light, would most likely render them biologically inactive presumably by preventing the strands of the DNA molecules from separating during replication. The experiments of Meselson and Stahl[12] point to this mechanism of replication. Since Wang[24] has also shown that uracil can be induced to form dimers when irradiated in the frozen state, it is possible that the irradiation of nucleic acids *in vivo* would cause the formation of mixed uracil-thymine dimers. Such a phenomenon might cause the formation of inseparable RNA-DNA complexes, impairing RNA biosynthesis and function. The formation of cross-links between the complementary strands of the DNA molecule by the dimerization of homologous base residues might also explain the inability thus far to renature the UV induced nonhydrogen bonded lesions. The dimerization of the bases in the denatured regions of the DNA molecule would tend to distort the structure and prevent all the pairing of the Watson-Crick bases in the same register as existed in the native molecule.

It would appear, however, that interchain cross-link formation and the prevention of strand separation under biological conditions cannot alone account for the UV induced loss of the transforming activity of *D. pneumoniae* DNA. Other lethal events, such as intrachain links, photochemical alteration of the cytosine residues, localized denaturation of the double stranded structure, and some backbone breakage, etc., most likely take place. Cross-linking between *complementary strands* cannot explain the UV inactivation of the phage ϕX 174[30] whose DNA has been shown to be single stranded.[31] It is apparent from the results described in this communication that the amount of UV damage detected by the use of the *E. coli* phosphodiesterase (Figs. 4 and 5) is greater, for the same UV doses, than is estimated by the use of the CsCl density-gradient technique (Figs. 2 and 3). This discrepancy is easily resolved assuming the existence of *intra*chain cross-links or other lesions which are induced by the UV irradiation of native DNA. Such lesions, undetected by the CsCl technique, may be more apparent when examined by the enzymological method. If the main action of the photoreactivating enzyme is to disrupt the denaturation resistant cross-links, which it carries out in a very efficient manner (Fig. 5), it is apparent that other photochemical lesions exist since this enzyme maximally reactivates UV treated transforming DNA from *Hemophilus influenzae* to 50 per cent of its original activity.[18] Experiments are now in progress to detect such nonphotoreactivable lesions and to extend our observations on the formation of denaturation resistant cross-links and to correlate this with dimer formation and biological inactivation. It is tempting to postulate that the UV induced mutations arise from the inability of the linked bases to pair properly during replication.

Summary.—The irradiation of DNA in solution with UV light with doses approximating those used for the inactivation of transforming activity causes (1) the irreversible hydrogen-bond breakage, most likely in internal regions of the DNA molecule and (2) the formation of formamide- and heat-stable linkages between the two complementary DNA strands. Repair of the latter damage is catalyzed by the photoreactivating enzyme from bakers' yeast. The cross-links are in keeping with the observations of others on the irradiation of thymine in the frozen state and may be due to the formation of dimers of thymine.

Note added in proof: P^{32}-DNA, which had been UV irradiated and denatured at room temperature with formamide, was hydrolyzed to the same extent as the heat denatured samples described in Figure 4.

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*ADENOSINE TRIPHOSPHATASE IN THE MITOTIC APPARATUS**

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The venerable and provocative speculation that all forms of movement in biological systems have a common molecular basis can, at present, be subjected to few kinds of experimental test, for all of which muscle contraction provides the paradigms. One test is the search for proteins corresponding to actin and myosin in their physical properties, a second is the preparation of working "models,"[1] and a third is the demonstration that the molecules composing the working structure not only interact with but split ATP.[2]

The unifying hypothesis concerning biological motility has included the movement of chromosomes in mitosis; one of the earliest speculation about this movement viewed the spindle fibers as functioning in a way that was comparable to the contraction of a muscle.[3] In the present study, which was motivated by the hypothesis, we describe the ATPase activity of the mitotic apparatus.

Although the isolated mitotic apparatus has been available for chemical study for some time,[4] the preparations provided by the earlier methods of isolation showed no ATPase activity. This we can now attribute to the employment of ethyl alcohol and of detergents in the isolation methods. The mitotic apparatus isolated by a new method,[5] which avoids these reagents contains an active and characteristic ATPase.

Methods.—Isolation of the mitotic apparatus: The principle of the new method has been described in several publications, and a detailed account of the procedure will appear shortly.[5] Sea urchin eggs (*Strongylocentrotus purpuratus*) are the source of material, providing large quantities of cells in synchronous division. The cells are fertilized, the extraneous membranes are removed, and the eggs are permitted to proceed into mitosis. At the appropriate stage, they are suspended in a medium consisting of 1.0 *M* sucrose, 0.15 *M* dithiodiglycol, and 0.001 *M* EDTA. In this medium, they may readily be dispersed by gentle mechanical agitation, freeing the mitotic apparatus. Further washing by centrifugation in the same medium removes those cytoplasmic components which are not contained in the mitotic apparatus.

Solution of the mitotic apparatus: The method outlined above isolates the entire mitotic apparatus; spindles, asters, centers, chromosomes, as well as yolk particles, mitochondria and fat droplets which are normally embedded in the periphery of the asters between the "rays," and submicroscopic particles which are contained in the matrix of the spindles and asters. In short, we isolate a *region* of the dividing cell, rather than a particular class of structures. While it is of interest to know the chemistry of the entire region in which the mitotic operations are taking place, we are particularly interested in defining the chemical properties of the characteristic "fibrous" system of the spindles and asters. To examine this, we have employed two further selective treatments following the isolation and purification of the whole mitotic apparatus.

(1) The apparatus are concentrated by centrifugation from the sucrose-dithiodiglycol-versene medium and then dissolved in 0.53 *M* KCl at pH *ca.* 8. This medium is isotonic to the original cellular medium and should preserve yolk particles, mitochondria, and other particles against osmotic lysis. In fact, we can observe under the microscope the dissolution of the mitotic apparatus in 0.53 *M* KCl, liberating intact yolk particles, chromosomes which sometimes remain associated in metaphase and anaphase groupings, the central regions of the asters, and other particles. These particles were removed by centrifugation at 25,000 *g* or higher for 15 minutes or longer, and the supernatant was regarded tentatively as a solution of the structural components of the spindles and asters. The activity of this solution was not reduced by centrifugation at 100,000 *g* for up to 2 hours.

(2) A converse procedure was based on the treatment of the mitotic apparatus, after isolation, with 10^{-2} *M* $MgCl_2$. In this medium, the fibrous structure of the spindles and asters was irreversibly stabilized, the chromosomes were preserved, but the large particles were "exploded" by osmotic forces and the small particles appeared to be washed away. After several washings, a preparation looking like a structural skeleton of a mitotic apparatus was recovered. For enzyme assays, a suspension of these residues of the mitotic apparatus could be used.

ATPase assays: The following modification of a method described by Perry[6] was used. The incubation mixture contained 0.5 ml of 0.2 *M* glycylglycine buffer (8.4), 0.15 ml of 0.05 *M* $MgCl_2$, 0.15 ml of 0.02 *M* ATP (Pabst), and 0.5 ml of the enzyme solution, made up with water to a final volume of 1.5 ml. The system was incubated at 24°C for one hour. The reaction was stopped by the addition of 1 ml of 5 per cent perchloric acid. A 1 ml aliquot was used for estimation of inorganic phosphate. For each assay, three blanks were used: one containing no ATP, one containing no enzyme, and one containing neither enzyme nor ATP.

Phosphate determinations were made by a modification of the method of Pons and Gurthrie.[7] To each 1 ml aliquot of the reaction mixture, 1 ml of 1.25 per cent ammonium molybdate in 0.75 *N* H_2SO_4 was added. After shaking, 1 ml of isobutanol was added and the mixture shaken again. A 0.5 ml sample of the isobutanol phase was removed from each tube and mixed with 0.4 ml of fresh $SnCl_2$ solution. After 20 minutes, 5 ml of 95 per cent ethanol were added, and the color read at 660 μ.

Enzyme activity was computed on the basis of Kjeldahl nitrogen determinations on the enzyme samples.

Results.—The mitotic apparatus does possess an ATPase activity which remains in solution when the isolated preparations are dissolved in 0.53 *N* KCl and cleared of particles, and which remains associated with the structure when it is stabilized in 10^{-2} *M* $MgCl_2$.

In Table 1, the results of measurements on a series of preparations, at pH 8.4 and in 5×10^{-3} *M* Mg^{++} are listed. It is seen that the activity recovered is variable and that it is higher in the apparatus stabilized by Mg than in the KCl solutions.

TABLE 1

ATPASE ACTIVITY OF KCl-SOLUBILIZED AND MAGNESIUM-STABILIZED PREPARATIONS OF THE MITOTIC APPARATUS IN $\frac{\text{m}M\ \text{Pi}^* \times 10^{-6}}{\mu g\ N \text{ per hr}}$

KCl-solubilized	Magnesium-stabilized
3.74	5.90
2.16	4.01
1.68	5.35
2.51	...
3.43	...
2.96	...
3.51	...
1.71	...
Av. 2.80 ± 0.81	Av. 5.08 ± 0.94

* Pi = inorganic phosphate liberated from ATP.

The following characteristics of the enzyme have been determined. (1) The pH dependence shows a broad optimum between 7.8 and 8.6. (2) The activity is dependent on divalent cations, Mg^{++} being the most effective. Activity in the presence of Mn^{++} is 70 per cent that in the presence of Mg^{++}, both being at 0.005 *M*. Activation by Ca^{++} was only about 33 per cent as effective as activation by Mg^{++}, again at 0.005 *M*. There was no demonstrable competitive inhibition by Ca^{++} in the presence of Mg^{++}. (3) The activity is specific for ATP. The splitting of inosine triphosphate was only 39 per cent that of the splitting of ATP. Guanosine triphosphate, cytidine triphosphate, and uridine triphosphate were not

split at all. (4) No pyrophosphatase, alkaline phosphatase, or ADP-splitting enzymes was found.

The ATPase activity was recovered from the supernatant fraction after the mitotic apparatus was dissolved in 0.53 *M* KCl and centrifuged at 105,000 *g* for 2 hours. This argues against the possibility that the activity was associated with the cytoplasmic particles trapped in the mitotic apparatus rather than with the "fibrous" component that is dispersed by treatment with KCl. The same conclusion is drawn from the fact that the activity was not extracted by KCl solutions after the mitotic apparatus was stabilized with Mg^{++}. These arguments are not absolutely convincing, and a final test of the association of the ATPase with the molecules responsible for the "fibrous" structure of the mitotic apparatus will depend on the isolation of these molecules.

Summary.—The idea that "working" molecules have the properties of ATPases has been a central theme in the study of the biochemistry of motility, even though no concensus exists even in the field of muscle biochemistry as to the role of ATP or of the splitting of ATP. In the present study, the existence of a highly specific ATPase in the mitotic apparatus has been demonstrated. This activity is confined to the splitting off of the terminal phosphate of adenosine triphosphate; GTP, CTP, and UTP are not substrates for it. The presence of the enzyme in the mitotic apparatus allows the hypothesis that ATP is concerned with the mechanochemical transduction by which the chromosomes are moved. The evidence favors the conclusion that the ATPase activity is associated with the fibrous structural components of the mitotic apparatus; it becomes soluble when the apparatus is dispersed in 0.53 *M* KCl and remains in stable association with the structure when it is stabilized by Mg^{++} ions. This conclusion can be verified only by a satisfactory isolation of the molecules responsible for the fibrous structure.

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*TAUTOMERIC FORMS IN A POLYNUCLEOTIDE HELIX AND THEIR BEARING ON THE STRUCTURE OF DNA**

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Some years ago the author initiated a study of the infrared spectra of nucleosides and nucleotides with the purpose of determining which tautomeric forms of the nucleotides predominated in aqueous solution.[1, 2, 2a] The biological role of DNA as the chemical repository and transmitter of genetic information confers upon this structural feature of the component bases a particular importance in molecular biology. Hydrogen bonds are known to contribute a large part of the energy which stabilizes and holds together the strands of the nucleic acid helices. From the viewpoint of nucleic acid structure it is thus clearly important to know which atoms have the hydrogens attached to them, since only those hydrogen bonding systems can exist which are compatible with the tautomeric forms present in the nucleic acids. It might be expected, therefore, that convincing evidence on tautomeric forms in aqueous solution could provide a criterion for deciding between certain alternative nucleic acid structures.[1] This possibility has been given present pertinence by a recent study of Hoogsteen,[3] who has shown that 1-methylthymine and 9-methyladenine co-crystallize in the unexpected hydrogen bonding scheme XII (see formulas below). This work and the suggestion[4] that it provides, "a strong indication that an arrangement differing from that of Watson and Crick is involved in nucleic acids...," have given added stimulus to the infrared studies in the hope that they might provide a criterion for distinguishing between two crystallographically possible nucleic acid structures. A note proposing bonding scheme XII specifically for an alternative DNA model has recently appeared.[4a] The application of tautomeric form as a criterion of structure is discussed in a later section.

It may further be noted that the suggestion of Watson and Crick[5, 6] that an infrequent change to a less stable tautomeric form may be the specific chemical change responsible for spontaneous mutation clearly adds interest to the investigation of this chemical property. The extreme sensitivity of the genetic test, however, will obviously present a challenge to chemical or physical methods which may be designed to approach it in sensitivity.

In pursuing the tautomeric studies it was a matter of the first importance to deal with aqueous solutions because of the great difference in physical and chemical properties between the usual organic solvents and the biologically important solvent, water. A further requirement for obtaining information of biological importance is that the method employed should be applicable not only to the mononucleotides but also to the nucleic acids themselves.

Although the highly sensitive tool of ultraviolet spectroscopy is amenable to work in aqueous solution, the similarity of the spectra of many of the nucleotides and the overlap of the component bands to produce single peaks in the nucleic acids make its application to this aspect of nucleic acid structure unreliable.

The determination of tautomeric equilibria of aminopyridines from ratios of dis-

sociation constants of alkylated pyridines has been discussed by Angyal and Angyal.[7] This method is not useful for present purposes, however, even if the assumptions upon which it is based introduce no errors, because the multiplicity of ionizable groups in the nucleic acids precludes the deduction of tautomeric form from the titration curves.

Although the author originally suggested the possibility of employing nuclear magnetic resonance to determine tautomeric structure of the nucleotides,[1] it appears that the method is incapable of supplying this information for nucleotides in neutral solution or for the nucleic acids. The exchange with the solvent prevents the observation of peaks caused by the tautomeric hydrogens,[8] though the nondissociable hydrogens of the nucleotides can be readily observed. A nuclear magnetic resonance study of DNA in water solution has yielded important information about the influence of the DNA upon the solvent,[9] but the hydrogens of the macromolecule, whether dissociable or not, cannot be observed.

Infrared absorption, being directly related to the vibrations of the atoms in the molecules, is responsive to changes in molecular structure in a manner which often closely reflects the chemical properties of the molecules. The infrared spectra of the nucleotides are characteristic, usually complex, and sufficiently different from each other to permit resolution of absorption bands in appropriate mixtures. This last property is important if the question of possible change in tautomeric structure upon helix formation is to be dealt with, and it must be dealt with if the information is to be reliably applied to the nucleic acids themselves.

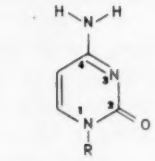
In spite of these potential advantages of infrared spectroscopy, however, the very strong absorption of water rules out the use of this solvent in those regions of the spectrum which are structurally most interesting and useful, i.e., those corresponding to the stretching vibrations of most single and double bonds. Gore *et al.*[10] first pointed out that this difficulty can be circumvented by the use of D_2O, which has a window in the region of double bond absorption.[10a] Sinsheimer *et al.*[11] took advantage of the transparency of D_2O as an infrared solvent in a valuable study of the spectra of a number of pyrimidine nucleotides at different pH values and over a wide range of frequencies. The method of varying the pH, however, while very informative in some respects, is not capable of providing reliable evidence of nucleotide tautomeric structure. When the heterocyclic bases are electrically charged by the loss or addition of a proton, there may be important changes in the electronic distribution in those bonds whose vibrations are responsible for the absorptions. Those views which were expressed[11] on tautomeric structure were generally based on analogy to other methods rather than on the solution spectra themselves. In the case of cytidine the authors preferred the imino form for the neutral molecule and the ammonium form (additional proton on the nitrogen external to the ring) for the cation. In the present paper we shall submit evidence favoring rather the amino and immonium forms (protonation of the ring nitrogen).[11a]

Blout and Lenormant have also employed D_2O to observe various biological materials[12] and highly polymerized DNA.[13] The spectra were too complex, however, to permit unambiguous interpretation on either the macromolecular or the monomer scale, and no tautomeric forms could be assigned.

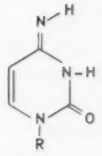
It is clear that an understanding of the spectral properties of the polymers must

be based on an understanding of the spectral characteristics of the mononucleotides. For this purpose a number of water-soluble N- and O-alkyl model compounds were prepared.[1, 14, 15]

The structures which are important for the present study are shown below (R = methyl for structure IV; R = glucosyl for III; and R = ribosyl for the other structures).



I



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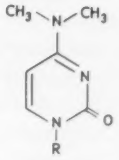
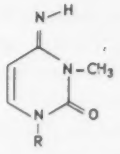
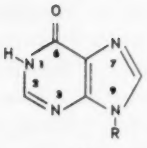


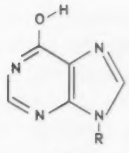
III



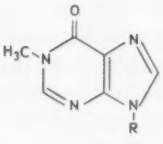
IV



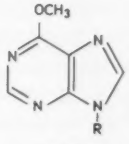
V



VI



VII



VIII

The labile hydrogens of the nucleosides could be attached to either of two nitrogens in cytidine (cf. I and II) and to either oxygen or nitrogen in inosine (cf. V and VI).[16] The function of the alkyl groups of the models is to fix these compounds in structures which are isoelectronic with the possible tautomeric forms of the corresponding nucleotides. When the double bond arrangement is then determined by comparison of the spectra, the position of the hydrogen is uniquely determined by the laws of valence.

It was found that the spectrum of cytidine (I or II) in the region of double bond absorption was virtually the same as that of the model compound III and quite different from that of IV (for details of the spectra, see the following sections), thus establishing the structure of cytidine as the amino form I rather than the imino form II.

Similarly, inosine was shown to exist in the keto form, V, because of the close similarity of its spectrum with that of VII and its wide divergence from that of VIII.

After the discovery of the homopolynucleotides[17, 18] and of their interactions[19–24a] it became possible to apply the earlier spectral work on the monomers[1] to the determination of the tautomeric forms which exist in the polynucleotide helices.[2, 2a]

The polynucleotide interactions (A + U) and (I + C) were observed in the infrared,[2, 2a] and with the models available at the time, it was possible to make definite assignments of the tautomeric forms of two of the four bases and probable assignments of the other two. In the case of poly (I + C) it was shown that the inosine units possessed the keto structure both before and after interaction,[2a] in contrast to a previous proposal,[25] based on ultraviolet studies, that the tautomeric form of the inosine units changed upon interaction of poly I with poly C. In the

present paper evidence is presented that the cytidine units exist in the amino form. The possibility of observing the interaction of the polynucleotides by pairs has thus been invaluable in this, as in other studies, in permitting a separation of variables which were too numerous and too complex to be dealt with convincingly in the nucleic acids themselves.

A more detailed discussion of the deduction of tautomeric form from the spectra and the application of this information as a criterion of nucleic acid structure is given in the following sections.

In addition to permitting the determination of tautomeric form the spectra have shown certain characteristic changes in the frequency and intensity of the absorption bands[2, 2a] as the helix is formed. The most prominent changes have been decreases in intensity and increases in frequency—both changes in the opposite directions from those to be expected simply on the basis of increased strength of hydrogen bonding. The author has proposed[26] that as a first approximation these changes are the resultant of shifts produced by two major effects: (1) stronger hydrogen bonding and (2) a decrease in dielectric constant of the immediate environment of the vibrating groups (caused by the forcing of water away from the surfaces of the planar bases as they are packed tightly in the helix). Spectroscopic studies[27] of small molecules have shown that a decrease of dielectric constant produces a decrease in intensity and increase in frequency of carbonyl bands, though more specific interactions may also make important contributions to the spectral changes. It may be expected that further studies of the origins of the infrared spectral shifts will permit an improved insight into the nature of the chemical interactions in the core of the nucleic acid helix.

It is clear that the spectral changes[13] observed on the disruption of the DNA helix must have the same origins as those discussed above and that, in principle at least, it should be possible to construct a nucleic acid spectrum from its component parts.

Results and Discussion.—(a) *Tautomeric form of cytidine and poly C:* It is seen that cytidine (I), the amino model (III), and poly C (Fig. 1, *A*, *B*, *K* resp.) all have essentially the same spectrum in the double bond region, with a strong band at 1651 ± 2 cm^{-1} and a weaker band at somewhat lower frequency (1618, 1625, and 1617 cm^{-1}, resp.). The imino model (IV)[27a] (Fig. 1, *C*), however, has a markedly different spectrum with a strong band at 1657 cm^{-1} and moderate to strong bands at 1671 cm^{-1} and 1579 cm^{-1}. These spectra are believed to establish beyond any reasonable doubt that cytidine and poly C have the amino rather than the imino form in solution.

It is considered probable, though not essential to the above argument, that the strong bands at 1651 ± 2 cm^{-1} (Fig. 1, *A*, *B*, *K*) are caused to a large extent by the vibration of $C_2 = O$ bond and that the weaker bands at 1618 cm^{-1}, 1625 cm^{-1}, and 1617 cm^{-1} have a large contribution from C = N vibrations in cytidine, III, and poly C, respectively. It is not necessary to consider NH_2 deformations, which fall in the same general region, because the solvent is D_2O rather than water and because replacement of NH_2 with $N(CH_3)_2$ does not cause the disappearance of a band.

It is suggested that the 1657 cm^{-1} band of the imino model (IV) may be attributed largely to a C = O vibration and that the 1671 cm^{-1} absorption may

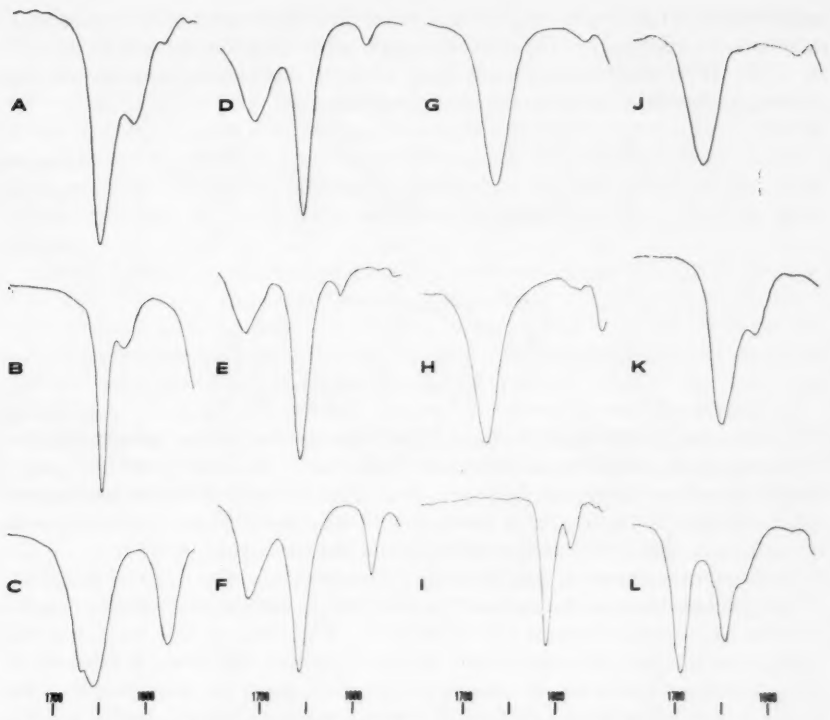


FIG. 1.—Infrared absorption spectra from 1550 cm^{-1} to 1750 cm^{-1} in D_2O solution. Abscissa, frequency of absorption in cm^{-1}; ordinate, per cent transmission on arbitrary scale. Conditions indicated in footnotes to Table 1. A, Cytidine; B, III (1-(β-D-glucopyranosyl-4-dimethylamino-2-pyrimidone). C, IV (1,3-dimethyl-cytosine). D, Acid solution of cytidine. E, Acid solution of III. F, Acid solution of IV. G, Inosine. H, VII (1-methylinosine). I, VIII (6-Methyoxy-9-β-D-ribofuranosylpurine). J, Poly I. K, Poly C. L, Poly (I + C).

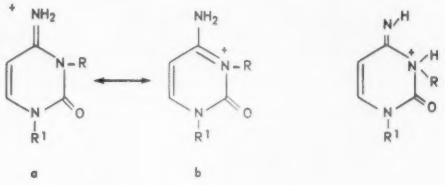
have a large contribution from a C = N vibration.

It was found that the result of changing the solvent from D_2O to chloroform is to increase the separation of the two strongest absorptions of IV (peaks at 1689 cm^{-1} and 1663 cm^{-1} in chloroform). With III in chloroform (R = tetraacetyl-β-D-glucopyranosyl) there is also an increase in the frequency of the two strongest bands (to 1657 cm^{-1} and 1638 cm^{-1}) but a decrease rather than increase in band separation.

The possibility that the difference between the spectra of III and IV is due to protonation of IV is ruled out by the quite different acid spectra in the following section and by the fact that the spectrum of IV was determined in neutral, buffered solution (pH 7.4.)

(b) *Tautomeric form of cytidine in acid solution:* The spectra of cytidine and of compounds III and IV (Fig. 1, *D*, *E*, *F*) are all nearly identical in acid solution, indicating that they have an isoelectronic[28] structure. It is the model compound, IV, which shows that this common cation must have the structure IX because this structure would be more stable than the alternative, X. IX (R = R′ = CH_3)

can be stabilized by resonance between the equivalent forms a and b, whereas X cannot be so stabilized. The cytidine cation must therefore have structure IX (R = H; R′ = ribofuranosyl) and hence have the additional proton on the ring nitrogen rather than on the external amino group.[11a]



a b

IX X

(c) *Tautomeric form of inosine and poly I:* Inosine (V: Fig. 1, *G*), poly I (Fig. 1, *J*) and 1-methylinosine (VII: Fig. 1, *H*) all have spectra with a single strong band in the region of double bond absorption (1673 cm^{-1}, 1677 cm^{-1}, and 1678 cm^{-1} resp.), suggesting a common keto structure. The radically different spectrum of the enol model (VIII: Fig. 1, *I:* bands at 1611 cm^{-1} and 1586 cm^{-1}) establishes the correctness of this interpretation and rules out the enol structure VI.[29]

(d) *Tautomeric forms in poly (I + C):* The spectrum (Fig. 1, *L*) of this interaction product shows a strong band at 1697 cm^{-1}, another at 1648 cm^{-1} and a shoulder of moderate intensity at 1630 cm^{-1}. The band at 1697 cm^{-1} is attributed to the inosine units (the reason for the shift from 1677 cm^{-1} is discussed in the introduction and in ref. 26) and shows that they are in the keto form since the N-alkyl model absorbs at 1678 cm^{-1} whereas the enol model absorbs at 1611 cm^{-1}, a region quite free of strong absorption in the poly (I + C) spectrum (Fig. 1, *L*). The remainder of the curve (Fig. 1, *L*) shows a close similarity in general shape and appearance to the curve of the amino model, III (Fig. 1, *B*) and of poly C (Fig. 1, *K*) and marked differences from the curve of the imino model, IV (Fig. 1, *C*). The following specific points may be observed: both major cytidine absorptions are present (at 1648 cm^{-1} and ~1630 cm^{-1} (sh.)) with approximately the same relative intensities, though with slightly shifted frequencies and with lower resolution.

Two of the absorptions characteristic of the imino form (1671 cm^{-1} and 1579 cm^{-1}; Fig. 1, *C*) are completely lacking in the poly (I + C) spectrum (Fig. 1, *L*). These spectra, therefore, lead to the conclusion that in this interaction product the cytosine units are in the amino rather than the imino form. It should be noted that this conclusion does not depend upon X-ray data, nor does it presuppose any particular helical model.

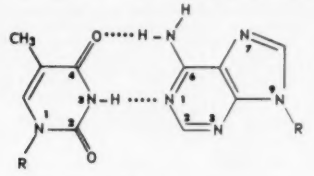
Nature of the helical interaction product, poly (I + C): The first report of the interaction of poly I with poly C indicated that a 1:1 complex was formed and that the fibers obtained from the interaction product had an X-ray diffraction pattern resembling that of RNA,[22] whereas the diffraction pattern of poly (A + U) had resembled that of DNA.[20]

Observations of the infrared spectra were employed to study the tautomeric form[28] and a preliminary attempt to obtain further information about the structure

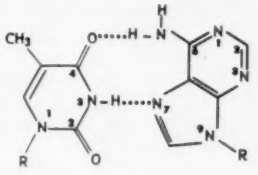
in solution was made by measuring the optical rotation of poly (I + C) and comparing it to that of poly (A + U). The author found that poly (I + C) had the same specific rotation as poly (A + U) $[\alpha]_D^{25°} = 290°$; $[\alpha]_{365}^{25°} = 1200°$; Doty *et al.*[24] report $[\alpha]_D^{25°} = 300°$ for poly (A + U)) and a similarly sharp decrease in optical rotation upon heating (melting-out curve).[30] These experiments suggested the possibility that poly (I + C) might, like poly (A + U), have a DNA-like structure in solution, though the possibility of drawing a definite conclusion from such evidence will have to await a better knowledge of the dependence of rotation upon structure.

In further experiments with poly (I + C) Davies has obtained highly crystalline fibers with an X-ray diffraction pattern characteristic of a DNA-like structure.[31] It thus appears that the poly (I + C) helix has a structure like that of DNA but that this structure could be composed either of a hydrogen bonding scheme of the Watson-Crick type (XIII) or of the alternative arrangement, XIV (see following section).

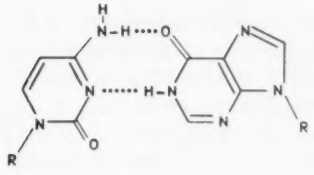
Application to the structures of poly (I + C) and of DNA: The structural model of DNA proposed by Watson and Crick[32] required the presence of the purines and pyrimidines in two specific complementary bonding arrangements, one between adenine and thymine (XI) and the other between guanine and cytosine (the addition of the third hydrogen bond as shown in XV follows the proposal of Pauling and Corey.)[33] The first specific proposal of a fundamentally different bonding arrangement in a case clearly applicable to the nucleic acids was that the second



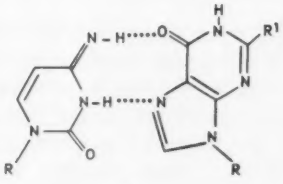
XI



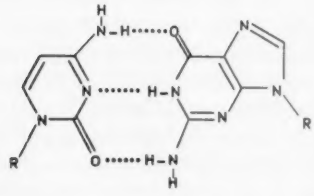
XII



XIII



XIV



XIV

strand of poly U in the three-stranded helix poly (A + 2U) was bonded to the N_7 position of adenine[34, 35] as shown in XII (the other poly U chain was believed to be bonded in the Watson-Crick manner, XI, at N_1 of adenine). It has since been found[3] that 1-methyl-thymine and 9-methyladenine co-crystallize in the arrangement XII rather than XI, and this has led to the proposal[4] cited in the introduction that an arrangement differing from that of Watson and Crick may be involved

TABLE 1

INFRARED SPECTRA IN D_2O SOLUTION[a]

	Compound	ν_{max} (cm^{-1})		
A	Cytidine	1650	1618	...
B	III[b] (amino model)	1649	1625	1548
C	IV[c] (imino model)	1671	1657	1579
D	Acid cytidine[d]	1712	1657	1591
E	Acid III[d]	1718	1658	1617
F	Acid IV[d]	1716	1658	1581
G	Inosine	1673	...	...
H	VII[e] (keto model)	1678	1555	...
I	VIII[f] (enol model)	1611	1588	...
J	Poly I[g]	1677	...	...
K	Poly C[g]	1653	1617	...
L	Poly (I + C)[g]	1697	1648[h]	~1630 (sh.)

[a] These qualitative spectra were measured on a Beckman IR7 grating-prism spectrophotometer in D_2O solution, solvent compensated (0.025 mm path length). The instrument was purged continuously with dried air, and for some of the spectra, high scale expansion was employed.

[b] See references 1 and 14.

[c] Prepared by the method of Hilbert[27a] and purified by crystallization and sublimation (mp 150°). This spectrum was measured in 0.1 *M* sodium cacodylate buffer, pH 7.4, and in unbuffered D_2O, the same result being obtained in each case.

[d] The cytidine solution was made approximately 0.1 *N* in acid by adding D_2SO_4. The crystalline hydroperchlorate[1] of III was used. The crystalline hydroiodide (called the methiodide by Hilbert because of its method of preparation) of IV and a sulfuric acid solution of IV (pH 2) were both measured and gave the same spectrum.

[e] Bredereck and Martini[42] reported that the product of the reaction of diazomethane with triacetylinosine was 1-methylinosine but made little serious attempt to purify or characterize the product. It has now been found that the reaction product, after ether precipitation as described, contains the 6-methoxy and 1-methyl derivatives as well as other substances. Pure 1-methylinosine has been obtained by column chromatography of the reaction mixture and found to be a crystalline substance of mp 209–210°, λ_{max} = 251 in H_2O. Details of the purification and characterization will be published at a later date.

[f] The author is indebted to Dr. H. Schaeffer for a sample of the O-methyl compound.

[g] The polynucleotides used were the very generous gift of Dr. Marie Lipsett and Dr. Leon Heppel. The solutions were 0.05 *M* in sodium chloride and 0.01 *M* in sodium cacodylate buffer, pH 7.0. The poly I was versene-dialyzed and the poly C water dialyzed.

[h] The poly (I + C) spectra obtained in these experiments are essentially the same as those reported previously,[2a] but are of considerably higher quality because of the purging of the spectrophotometer in these experiments with dry air. At the high scale expansion used (90–100% T expanded full scale) atmospheric water vapor was not completely compensated in the earlier spectra. With these dilute solutions (about 0.009 *M* in bases) slow pen response and scanning speed are employed to minimize noise.

in nucleic acids. A recent note[4a] has proposed this bonding scheme, (XII), specifically for DNA, the cytosine units in the G-C pairs being in the imino tautomeric form, although the reason for the selection of the imino form was not stated. The authors proposed the alternative model[4a] for DNA but stated that it, "...introduces some stereochemical difficulties which make it less likely than the Watson-Crick model." Some pertinent aspects of the alternative bonding schemes and of the DNA model based upon them are discussed below.

It should be understood, as background for the subsequent argument, that a requirement of any two-stranded model for DNA is that there be equivalence of the glycosidic bonds of the base pairs (i.e., that it be possible to superimpose simultaneously both of the glycosidic bonds of successive base pairs; a more detailed statement of this condition has been given by Pauling and Corey).[33]

It is possible that the arrangement shown in XII could form the basis of a DNA structure which satisfied the above-mentioned principle of equivalence. But if this is the adenine-thymine pairing in DNA, then in order to preserve the equivalence of the glycosidic bonds of the base pairs, the guanine-cytosine bonding pattern

would have to be that indicated in XIV (R = NH_2).[36] A necessary feature of this structure is that the cytosine units must exist in the imino form. In the Watson and Crick bonding scheme the cytosine units are in the amino form.

The spectral evidence presented above demonstrates that in poly (I + C) the cytosines exist in the amino form, and, therefore, that the bonding scheme cannot be that shown in XIV (R = H). Further, since cytosine has been shown to have the amino form in cytidine, in poly C, and in the DNA-like helix, poly (I + C), it is most reasonable to expect that it will maintain in DNA the tautomeric form which has proved to be the more stable one throughout this range of molecular structures.

Having stated this conclusion, however, let us ask whether it is likely to be changed by a more explicit consideration of two obvious structural differences between DNA and the polynucleotide helices. The latter have hydroxyl groups on the C_2 atoms of the sugar residues where the former has hydrogen atoms. It was found in the case of poly (A + U) that this difference resulted in a slight increase in the diameter of the molecule,[25] but the arrangements of the bases deduced from the X-ray data[25, 31] indicate that this increase is not the result of an increased separation of the coplanar bases (if the separation of the bases were increased very much, of course, effective hydrogen bonds could not be made). It is concluded that the presence of an additional hydroxyl group on the sugar is quite unlikely to alter the tautomeric forms of the bases.

The second point may then be considered: is the difference between inosine and guanosine sufficient to result in a change in the tautomeric form of the cytidine units on going from poly (I + C) to DNA? It can be seen that in XIV the amino group (R′) by which the two bases differ is now remote from the cytosine and can no longer interact with it as in XV. We conclude that since the structure XIV (R′ = H) is not sufficiently stable to exist in preference to XIII, it is most unlikely that the presence of an additional amino group (R′ = NH_2) at a point remote from the area of hydrogen bonding would render XIV (R′ = NH_2) more stable than XV (for structure XIV the argument would be the same if this functional group should exist in the imino tautomeric form).

The following arguments from the stabilities of different base pairings are presented in support of the structural conclusion reached above on the basis of the criterion of tautomeric form. Pauling and Corey[33] have pointed out that a third hydrogen bond can be formed between guanine and cytosine (XV) and suggested that this conclusion strengthens the arguments of Watson and Crick concerning the role of complementariness of structure of the two DNA polynucleotide chains in the duplication of the gene. The additional hydrogen bond in XV might be expected to confer higher stability upon the GC pair than the two-bond AT pair (IX) possesses, and, in fact, the work of Marmur and Doty[37] has shown a linear increase in melting temperature of DNA samples as the GC content is increased. The most reasonable, although not the only possible conclusion, is that the source of this greater stability is the additional hydrogen bond possible in the GC pair. While it could be supposed that GC forms only two hydrogen bonds, and that this pair possesses greater stability in a helix because these hydrogen bonds are intrinsically stronger than those in the AT pair, serious doubt is cast on this possibility by the fact that poly (I + C) has a lower melting temperature than poly (A + U)[108].

Structure XIV ($R' = NH_2$) would thus require the loss of the unique possibility of forming three hydrogen bonds, which the GC pair possesses, and which is a presumed major source of its higher stability.

The equivalence of base ratios[38] is a fundamental chemical fact which has been of great importance to our understanding of the nueleic acids and one which should be accommodated by any model for DNA. In the present case, however, it provides no criterion for distinguishing between the two models since either pair of bonding schemes would satisfy this condition.

The crystallographic data on DNA gave evidence only of rough compatibility with the Watson and Crick model[32, 39, 40] at the time of its publication, but more detailed studies have greatly improved the agreement between the model and the X-ray data.[41, 42]

The DNA model of Watson and Crick is thus supported by a variety of positive evidence, and no seriously contradictory evidence has as yet been advanced. While the alternative model based on structures XII and XIV is also compatible with some of the experimental evidence, in our view the present experiments and deductions from them make this alternative model quite improbable.

* Abbreviations and definitions: Poly I, polyinosinic acid sodium salt;[17] poly C, polycytidylic acid sodium salt; poly (I + C), the interaction product of poly I and poly C; poly A, polyadenylic acid sodium salt; poly U, polyuridylic acid sodium salt; poly (A + U), the 1:1 interaction product of poly A and poly U; poly (A + 2U), the 1:2 interaction product[34] of poly A and poly U; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; AT, adenine-thymine; GC, guanine-cytosine. The tautomeric forms of the nucleosides are those structures which differ in the position of attachment of a labile or exchangeable hydrogen and consequently in the positions of the double bonds. Thus, for example, the amino form of cytidine has the structure I and the imino form, the structure II; the keto form of inosine has the structure V and the enol form, the structure VI. The numbering system used for the purines and pyrimidines is indicated in I and V.

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[10a] Evidence that information about the polynucleotides in D_2O also applies to water has been presented recently by H. T. Miles, *Biochim. et Biophys. Acta*, **43**, 353 (1960).

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[11a] Ultraviolet spectra and arguments from resonance stabilization have been presented by C. A. Dekker, *Ann. Rev. Biochem.*, **29**, 453 (1960), in support of the immonium form of acid cytidine.

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[14] Miles, H. T., *J. Am. Chem. Soc.*, **79**, 2565 (1957).

[15] For references to other applications of N- and O-alkylation of pyrimidines see, for example,

the bibliography of Ref. 1, L. N. Short, and H. W. Thompson, *J. Chem. Soc.*, 168 (1952), and papers cited in Ref. 27a. A number of mono-oxygenated purine derivatives have been prepared and studied by D. J. Brown, and S. F. Mason, *J. Chem. Soc.*, 682 (1957), (cf. Ref. 29).

[16] Other possible tautomeric forms (e.g., the one placing the labile hydrogen of inosine on N_3 instead of N_1) are not considered here because, for stereochemical reasons, they could not participate in nucleic acid structure. Other tautomeric forms can, of course, be treated in the same manner as those shown here.

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[27a] Although the structure of IV was proved by Hilbert, *J. Am. Chem. Soc.*, **56**, 190 (1934). by conversion to the known 1,3-dimethyl-5-bromouracil, there have been observed methyl group rearrangements under certain conditions with some purines and pyrimidines [cf. Lawley, *J. Chem. Soc.*, 593 (1960) and Brown, Hoerger, and Mason, *J. Chem. Soc.*, 4035 (1955)]. Such rearrangements are in reality probably ring openings and closures (cf. Discussion in *Chemistry and Biology of the Purines*, Ciba Symposium, p. 57, (1957), and, being conducted under different conditions from those of Hilbert, are unlikely to have occurred in his experiments. The importance of the structure of IV to the present study, however, makes it worth observing that the only possible alternative structure is the isomeric 1-methyl-4-methylamino-2-pyrimidone and that this compound has been prepared by an unambiguous method by Kenner, Reese, and Todd (*J. Chem. Soc.*, 855 (1955)) and found to have properties different from those of IV. The author has repeated the preparation of 1-methyl-4-methylamino-2-pyrimidone and found that it depressed the melting point of IV and had a different infrared spectrum, confirming the correctness of structure IV.

[28] That is to say, the structures are the same in those structural features which govern the distribution of the double bonds; there will be a similar electron distribution whether R = H or R = methyl, the important difference being that when R = H it is dissociable and when R = methyl it is not.

[29] The interesting study of D. J. Brown, and S. F. Mason, *J. Chem. Soc.*, 682 (1957), has reported the infrared spectra of a number of methylated purines in chloroform solution and in the solid state. Though the authors generally favor the ketonic form of the oxo purines, as far as the tautomeric form of inosine is concerned the evidence cannot be considered conclusive. The required evidence may be provided by the compounds V, VII, and VIII. Of these only V, (R = CH_3) was reported. The O-methyl and N-methyl compounds corresponding to VII and VIII, but with R = hydrogen, were reported. Since the tautomeric structure of the imidazole ring is not defined when R = H, however, the result can be applied to inosine only by assuming that the spectrum of the pyrimidine ring is independent of the imidazole ring with which it is conjugated. Further, as indicated earlier, it cannot be assumed that the tautomeric forms in organic solvents are necessarily the ones existing in the nucleic acids. The authors presented a number of valuable correlations and proposed that the strong absorption of 9-methylhypoxanthine (1679 cm^{-1} in

the solid state; 1711 cm^{-1} in chloroform) is caused mainly by a C = O vibration.

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*NUCLEOSIDEDIPHOSPHATASE ACTIVITY IN THE GOLGI APPARATUS AND ITS USEFULNESS FOR CYTOLOGICAL STUDIES**

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Cytochemical information regarding Golgi material is limited. Early staining methods suggested the presence of alkaline phosphatase activity in the Golgi substance of intestinal mucosa and other cells, and of acid phosphatase activity in the Golgi "zone" of epithelial cells and macrophages.[1] However, it is clear now that the integrity of both Golgi apparatus and acid phosphatase-rich granules associated with it[2] could not survive the acetone fixation and paraffin embedding then employed. Biochemical studies of Golgi fractions isolated from homogenates of epididymis have indicated the presence of acid phosphatase activity in the Golgi apparatus.[3] Electron microscopy established the presence in the Golgi fractions of numerous membranous arrays characteristic of the organelle, but a critical study of the purity of the fractions is needed, in view of the observations to be presented here, before attributing the acid phosphatase activity to the Golgi apparatus.

We are presenting evidence indicating that in epididymis, as in all but two of the many cell types studied, acid phosphatase activity is concentrated not in the Golgi apparatus but in small granules located in the Golgi region of the cell. From the effects of the detergent, Triton X-100, to be described here and from considerations

discussed elsewhere,[2] these granules may tentatively be considered as lysosomes, the cytoplasmic particles whose existence in liver was first postulated by de Duve and his co-workers.[4] We are further reporting the presence of nucleosidediphosphatase activity in the Golgi apparatus. On the basis of this activity a staining procedure has been developed which permits demonstration of the Golgi apparatus in frozen sections and in cell smears. The method is of sufficient resolution to permit visualizing the Golgi apparatus *sensu strictu*, i.e., the microscopic lamellar structures consisting of aggregates of submicroscopic membranes distinguishable in the electron microscope.[5–7]

Materials and Methods.—More than 25 different rat, mouse, and frog tissues, and onion root tips were used in these studies. Tissues were fixed in cold 4% formaldehyde and frozen sections were incubated for enzyme activity in a medium patterned after that of Wachstein and Meisel[8] which in turn is a modification of Gomori's acid phosphatase medium.[9] It is buffered near neutrality; it has activating bivalent ions; and it contains lead ions to trap the phosphate liberated from the substrate by enzymatic hydrolysis. For demonstration of acid phosphatase activity, sections were incubated in the Gomori medium[8] at pH 5.0 and employing as substrate sodium α, β glycerophosphate[9] or freshly prepared sodium β glycerophosphate, as suggested by Holt.[10] The enzymatic nature of the reactions reported was checked as usual, by use of sections heated at 90°C for 5 minutes, by omission of substrate, and by addition of inhibitors to the medium (0.01 *M* sodium fluoride for acid phosphatase, 0.01 *M* uranyl nitrate for both acid phosphatase and nucleosidediphosphatase). In all these instances, essentially no staining occurred in the sections.

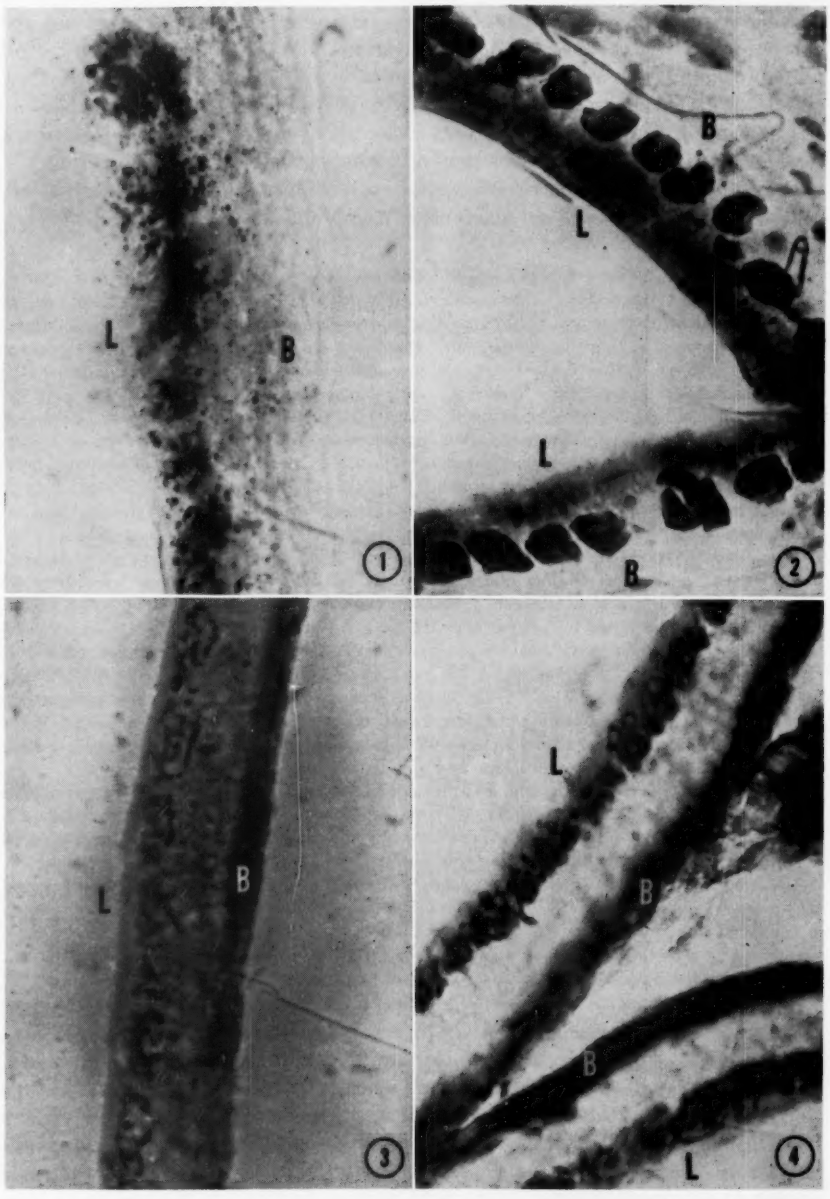
The substrates tested included the commercially available (Sigma; Pabst) tri-, di-, and monophosphates of guanosine, uridine, inosine, cytidine, and adenosine; thiamine pyrophosphate (Sigma); riboflavin phosphate (Sigma); and inorganic pyrophosphate (Merck). The last of these compounds was tested in the system of Naidoo and Pratt,[11] with manganese substituted for magnesium ions. Preliminary experiments were also done with diphosphopyridine nucleotide (DPN), flavin adenine dinucleotide (FAD), guanosine diphosphate mannose (GDPM), and uridine diphosphate glucose (UDPG). With these substrates, 3–5 mg of either acid phosphatase [wheat germ (Sigma)] or alkaline phosphatase [calf intestine (Worthington)] was added to the incubation medium, in order to liberate the phosphate residues from monophosphates resulting from any hydrolysis of the pyrophosphate bonds by the enzyme.

The effects of calcium, magnesium, and manganese additions were studied, as were variations in the pH of the medium. The effects of Triton X-100 were studied by treating sections with 0.25% solutions in distilled water for 5–60 minutes at 0°C and room temperature, rising them with several changes of cold distilled water to remove the Triton, and then incubating.

Tissue blocks or frozen sections of formaldehyde-fixed rat epididymis, rat brain (cerebrum), and the mouse Friend leukemia were incubated and then treated with osmium tetroxide and processed, in the usual manner, for electron microscopy. These experiments, performed by Dr. Edward Essner, will be reported elsewhere. Paraffin sections were made of all tissues except the onion root tips, following the Aoyama silver method for demonstrating the Golgi apparatus.[12]

Results.—Acid phosphatase activity: Except for rat testis and some cells of rat pituitary, acid phosphatase activity is not demonstrable in the Golgi apparatus by the methods employed. On the other hand, high levels of activity are localized in small granules which, in epididymis and many cell types, are concentrated in the Golgi region (Fig. 1). The lysosomal nature of the granules is suggested by the solubilizing effect of Triton X-100 (Fig. 2; cf. footnote 10). Where the acid phosphatase is localized in the Golgi apparatus (rat testis) the reaction product is present in nongranular form (at the light microscope level) in the lamellae. Furthermore, the acid phosphatase activity within the Golgi apparatus is far more resistant to Triton X-100 than that in the granules.

It should be noted that the α, β mixture has the advantage that the complete substrate medium may be stored in the cold for prolonged periods[9] whereas the β



FIGS. 1–4.—Frozen sections (7.5 μ) of rat epididymis fixed in cold formol-calcium.[19] For orientation purposes the bases of the cells are indicated by B and the luminal surfaces by L. Magnification, $\times 1100$.

FIG. 1.—Incubated in acid phosphatase medium of Gomori[9] for 10 min at 37°C. Note the individual black granules; these are presumed to be lysosomes.[2] Where the granules lie close together the precipitate of adjacent granules may merge, since the incubation time is long for these

medium deteriorates in about three days, as Holt[10] reported.

Nucleosidediphosphatase activity: In all cells thus far studied—normal and malignant, plant and animal—the lamellar Golgi structures show high levels of nucleosidediphosphatase activity (Figs. 3 and 5–8). The diphosphates of guanosine, inosine, and uridine are hydrolyzed much more rapidly than those of cytidine and adenosine. Sections of formol-calcium-fixed epididymis, cerebellum, duodenum, prostate, and seminal vesicle of the rat were used to establish optimal conditions for enzyme activity.[13] Activity is unchanged from pH 6.5 to 7.5. Manganese ions (1×10^{-2} to $1 \times 10^{-3} M$) are more effective and more specific in stimulating the Golgi enzyme (or enzymes) than magnesium ions; calcium ions stimulate minimally. The effects of these ions are the same in sections from tissues fixed in cold formaldehyde without added calcium, but preliminary studies indicate that in tissue fixed in formol-phosphate, magnesium ions stimulate as well as manganese ions. The possibility is currently being investigated that this is correlated with the difference in pH attained by the fixative (Table 1). Treatment with Triton

TABLE 1

FIXATIVES EMPLOYED IN CYTOCHEMICAL DEMONSTRATION OF GOLGI APPARATUS

Fixative*	Concentration of added bivalent ion	Initial pH	Final pH
Formol-calcium[19]	0.090 *M* Ca^{++}	6.9	5.6
Formol-calcium[20]	0.114 *M* Ca^{++}	7.9	7.1
Formol-cadmium[12]	0.044 *M* Cd^{++}	4.7	4.0
Formol-phosphate[20]	None	7.0	6.8
Formol, pH brought to 7.0 with NaOH	None	7.0	6.0

* All contain 4% formaldehyde and are used cold (0–2°C).

X-100, through the maximum time tested (1 hour at room temperature), does not solubilize the nucleosidediphosphatase activity in the Golgi apparatus (Fig. 4).

Nucleosidemonophosphates are not hydrolyzed in the Golgi lamellae; like glycerophosphate or riboflavin phosphate, they are hydrolyzed in granules, presumably lysosomes, that may be concentrated in the Golgi zone. In two of approximately 25 cell types studied (mouse plasma cell tumor MPC2, rat cerebellum) the Golgi apparatus (i.e., Golgi lamellae) appear to hydrolyze nucleosidetriphosphates.[13] Except for the plant cells studied (onion root tip), the Golgi lamellae of all cells tested also hydrolyze thiamine pyrophosphate (Figs. 5 and 6). Judged by intensity of staining, it is hydrolyzed almost as rapidly as inosine, guanosine, and uridine diphosphates. The effects of manganese, magnesium, and calcium are the same as with nucleosidediphosphates. Inorganic pyrophosphate is apparently not hydrolyzed in the Golgi apparatus under the conditions tested, even after 2 hours of incubation.

cells. Optical factors create an impression of larger masses in areas of the photograph.

FIG. 2.—Section incubated with the one shown in Fig. 1, but following treatment with Triton X-100 for 45 min at 0°C. As Holt[10] has shown with sections of rat liver and kidney, such treatment releases the acid phosphatase from the granules in epididymis and the many other cells we have tested. Note that: (1) few granules are now visible; (2) enzyme reaction product is spread diffusely in the "Golgi zone" and elsewhere in the apical cytoplasm; and (3) the nuclei are deeply stained, presumably due to adsorption of enzyme released from the granules.

FIG. 3.—Section incubated in IDP medium for 5 min at 24°C. The Golgi lamellae appear as threads (see footnote 5).

FIG. 4.—Section incubated with the one shown in Fig. 3 but following treatment with Triton X-100 for 45 min at 0°C. The nucleosidediphosphatase activity is undiminished (generally the stain is somewhat more intense) and the reaction product within the epithelial cells remains restricted to the Golgi lamellae.

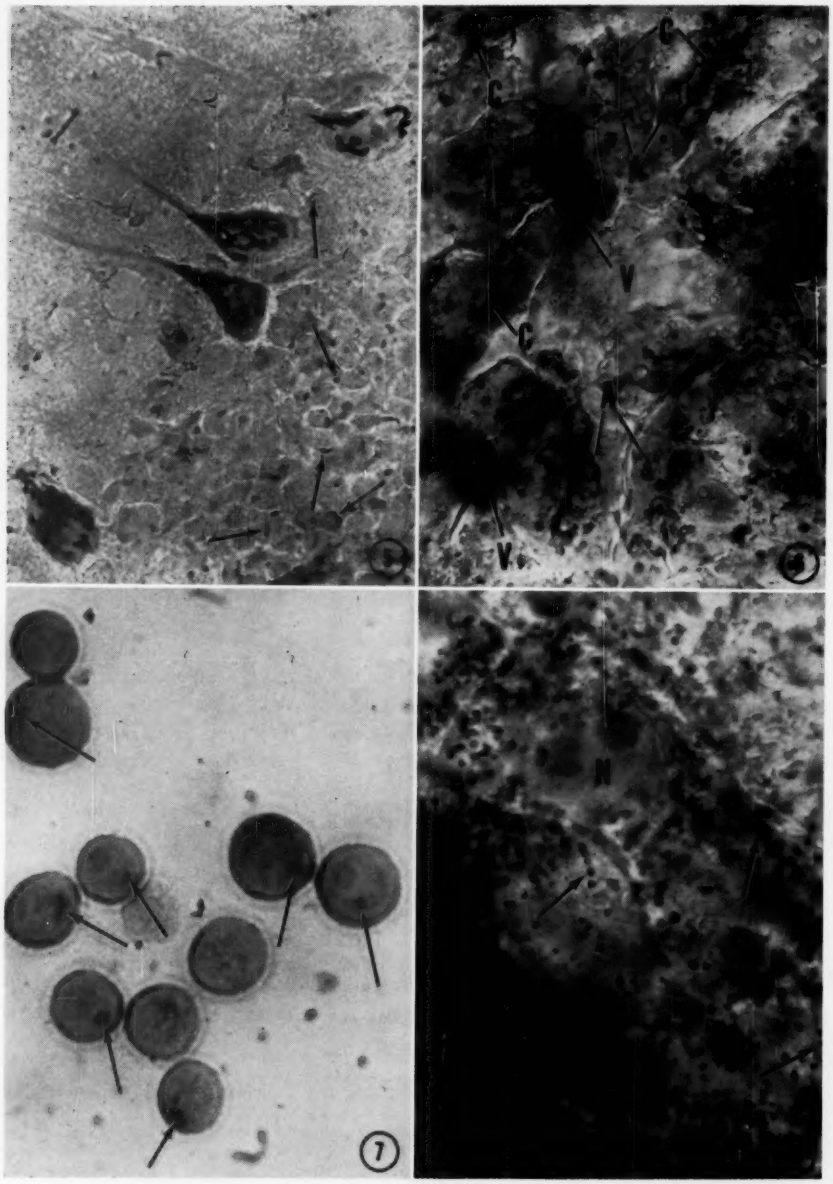


FIG. 5.—Frozen section (10 μ) of rat cerebellum fixed in cold formol-calcium and incubated, after nine weeks of storage of section in cold water, in TPP medium for 10 min at 37°C. Magnification, ×730. Note that in the large Purkinje cells the Golgi apparatus is extensively developed and has the "reticular" nature described by Golgi.[18] In the two neurons near the center of the field the Golgi lamellae may be seen extending into the dendrites. In the small neurons of the granular layer, the Golgi apparatus is small (arrows).

FIG. 6.—Frozen section (10 μ) of rat pancreas fixed in cold formol-calcium and incubated in TPP

Negative results were obtained in the preliminary attempts to demonstrate hydrolysis of compounds in which the nucleosidediphosphates are contained within the molecule (DPN, FAD, GDPM, UDPG). However, it has not yet been ascertained that the acid phosphatase or alkaline phosphatase added to the medium would hydrolyze orthophosphate from any monophosphate liberated by the hydrolysis of these compounds, and positive results in the staining procedure used depend upon trapping of orthophosphate as lead phosphate.

Correspondence of nucleosidediphosphatase reaction product with lamellae of Golgi apparatus: By sectioning sufficient numbers of paraffin blocks it was possible with all tissues processed by the Aoyama silver method to find areas of the sections in which the Golgi lamellae could be resolved. The appearances of the Golgi apparatus in these areas corresponded very well to those readily obtained in frozen sections by the cytochemical method based on nucleosidediphosphatase activity.

The precise nature of localization of nucleosidediphosphatase reaction product in the sections was emphasized by electron micrographs. Dense lead phosphate deposits were found in or between the paired Golgi membranes. None was found in the interspaces between adjacent membrane pairs or in any other cell organelle.

Discussion.—The results presented suggest the need for re-evaluating some interpretations drawn from biochemical analyses of Golgi fractions isolated from rat epididymis homogenates.[3] Until the distribution in the fractions of the acid phosphatase-rich granules (which are here described and which probably correspond to the lysosomes of liver and kidney[4]) is established, the extent to which the acid phospoatase activity of the fraction should be attributed to the Golgi apparatus will remain unknown. On the other hand, the need is evident for studying the effects of cold formaldehyde fixation upon the acid phosphatase and nucleosidediphosphatase activities of tissues and to assay isolated Golgi fractions for nucleosidediphosphatase activities. Such studies are in progress, some in the laboratory of Dr. Edward L. Kuff and some in our own.

The hydrolysis of both nucleosidediphosphates and thiamine pyrophosphate in the Golgi lamellae of animal cells does not establish the identity or plurality of the phosphatases involved. Our observations show always a similar behavior with both substrates: the identical patterns of cytological distribution in all cells, except in onion root tips; the same response to added bivalent ions in the medium; the same response to prior treatment of sections with Triton X-100; and the same response to the addition to the medium of $10^{-2}M$ sodium fluoride (no effect) and of $10^{-2}M$ uranyl nitrate (inhibition). However, to establish that a single enzyme is involved would require its isolation in pure form.

Comparing the Golgi nucleosidediphosphatase with similar enzymes reported in

medium for 20 min at 37°C. Magnification, ×940. The fine thread-like Golgi apparatus is seen at A. The larger dark structures, out of focus, are blood vessels (V). The "secretory capillaries"[21] are barely evident (C), and the zymogen granules, lying between "secretory capillaries" and Golgi apparatus, are not visible in the photograph.

FIG. 7.—Air-dried and formol-calcium-fixed cell of Novikoff ascites hepatoma of the rat, incubated in the IDP medium for 20 min at 37°C. Magnification, ×780. The Golgi apparatus (arrows) is situated near the identations of the nuclei,[22] barely visible in the photograph.

FIG. 8.—Frozen section (10 μ) of formol-calcium-fixed onion root tip, incubated in IDP medium for 20 min at 37°C. Magnification, ×1900. The cell outlines are barely perceptible, but the nuclei (N) are readily seen. The small dark bodies are consistent in size, number, and distribution with the multiple Golgi apparatus described in such cells by electron microscopists.[23] Arrows indicate some linear arrays, consistent with their presence in strands of streaming cytoplasm *in vivo*.

the biochemical literature is worthwhile. In 1950 Kornberg and Pricer[14] isolated a nucleotide pyrophosphatase from potatoes. It rapidly split DPN, TPN, and FAD, which we have thus far been unable to show to be hydrolyzed by the Golgi enzyme. However, the staining conditions are far from favorable for showing the hydrolysis of such compounds (see p. 807). The Kornberg and Pricer enzyme hydrolyzed thiamine pyrophosphate and adenosine diphosphate, as the Golgi enzyme does. On the other hand, it also hydrolyzed adenosine triphosphate. It was stimulated by magnesium or calcium ions but not by manganese ions, which gave a 9-per cent inhibition. In 1955 a nucleosidediphosphatase that closely resembles the Golgi enzyme was isolated from pork kidney cortex by Gibson *et al.*[15] and from beef liver by Plaut.[16] The Plaut enzyme hydrolyzed nucleoside diphosphates but not nucleoside tri- and mono-phosphates. Inosine, guanosine, and uridine diphosphates were cleaved, but apparently thiamine pyrophosphate was not studied. Optimal magnesium concentration and pH were similar to those of the Golgi enzyme; the effects of manganese and calcium ions are not mentioned. In contrast to the Golgi enzyme (or enzymes), the Plaut enzyme did not hydrolyze adenosine and cytidine diphosphates. When we stained rat liver sections for nucleosidediphosphatase activity, intense staining of the basophilic material occurred with inosine, guanosine, and uridine diphosphates but no staining was observed with adenosine and cytidine diphosphates. Since the basophilic material would be recovered largely in microsome fractions isolated from homogenates, we studied the distribution of the enzyme activity in subcellular fractions. The highest enzyme concentration was found in the microsome fraction. These results will be reported elsewhere. It should be noted, however, that some conditions abolish the staining due to the nucleosidediphosphatase activity in the basophilic material but do not affect the staining due to the Golgi enzyme.

Since the enzyme (or enzymes) in the Golgi lamellae of fixed cells apparently does not hydrolyze inorganic pyrophosphate and nucleoside monophosphates, and since it does not generally hydrolyze nucleosidetriphosphates, we have referred to it as nucleosidediphosphatase.

Light microscopic investigations of the topographical relations between the Golgi apparatus and acid phosphatase-rich granules in a variety of tissues[17] and appearances of electron micrographs in the literature[2] suggest a developmental or functional interrelation, in many cells, between lysosomes and the larger vacuoles surrounded by Golgi membranes ("Golgi vesicles" or "Golgi sacs").[6, 7]

Beyond the opportunity it provides for studying lysosomes and other intracellular organelles in the same or parallel sections or smears of fixed tissues (Figs. 1–4), the method here described affords other advantages. All methods for demonstrating the "internal reticular apparatus" first described by Golgi[18] have heretofore depended upon the penetration of reagents containing heavy metals (osmium or silver) into blocks of fixed tissue and the deposition of the metal within or upon the organelle. The slow rate of penetration of reagents makes this a lengthy procedure and it results in marked variability of staining at different depths of the blocks. The appearance of the Golgi apparatus varies with the extent of metal deposition upon it and upon adjacent structures. Such variability is unfortunate since functional significance is frequently attached to changes in apparent morphology of the apparatus. In contrast, the method here described is thoroughly re-

producible and yields the same staining picture in all parts of the section, thus largely eliminating the subjective element in comparative studies. The method is rapid, and yields preparations with excellent structural detail (Figs. 3–8). Most important, the method yields cytochemical information that may provide fruitful leads in the investigation of the specific biochemistry of this ubiquitous cell organelle.

Suggested Fixation and Staining Procedure.—1. Tissue blocks, 1–4 mm thick, are fixed overnight in cold 4% formaldehyde. Smears are rapidly air-dried and fixed in cold fixative for $1^1/_2$–2 minutes. Most of our work has been done with cold formol-calcium of Baker,[19] but we find both Golgi structure and enzyme activity equally well preserved in the fixatives listed in Table 1 which gives their pH and bivalent cation content.

2. After fixation, tissues may be rinsed in distilled water and sectioned. When necessary to improve the cutting quality of the tissues or when desirable to store the tissues, the sucrose-gum acacia procedure of Holt[10] may be followed. The washing may be prolonged, as Holt suggests, or reduced to several changes of cold distilled water over a period of 1–3 hours or less. They may be kept in cold 30% sucrose–1% gum acacia for 1 day to many weeks. Frozen sections (generally 5–10 μ thick) are cut, into cold distilled water, with a routine freezing microtome.

3. Sections may be used as soon as prepared or may be stored in a "section bank" from which they are subsequently drawn. Sections have been stored in cold distilled water for weeks without apparent loss of nucleosidediphosphatase activity of the Golgi apparatus (Fig. 5). The effects of such storage on a variety of enzyme activities will be reported separately.

4. Free-floating sections are incubated, for 3–30 minutes at 37°C or at room temperature, in either inosine diphosphate (IDP) or thiamine pyrophosphate (TPP) medium. At present, the cost of IDP is less than that of uridine or guanosine diphosphate. TPP is much less expensive and may be used for most cells. In the case of TPP, and of IDP when the substrate concentration or incubation time is increased, a precipitate forms in the medium. The precipitate may settle upon the sections and it may reduce the staining intensity. It should be removed by centrifuging the medium prior to use. When negative results are obtained with the substrate concentration indicated below, its concentration should be increased threefold.

5. The incubation medium consists of:

IDP (sodium) or TPP (chloride), 0.01 *M* in water	:	0.5 ml
Distilled water	:	0.2 ml
Tris-maleate buffer, pH 7.2, 0.2*M*	:	1.0 ml
1% Lead nitrate (0.03 *M*)	:	0.3 ml
Manganese chloride, 0.025 *M*	:	0.5 ml

When preparing TPP (chloride) solution for storage in the frozen state it should be brought to pH 6.5–7.0 with dilute alkali. This is unnecessary for IDP (Na) which gives a solution of pH 6.6.

6. Following incubation the sections are rinsed in distilled water, floated in dilute ammonium sulfide solution, rinsed and mounted in dilute glycerine or Kaiser's glycerogel, in the usual manner.[9, 20]

Summary.—In all cells tested the Golgi lamellae show high levels of hydrolytic activity, at neutral pH, toward nucleosidediphosphates (inosine, guanosine, and uridine diphosphates are split much more rapidly than cytidine and adenine diphos-

phates). In the animal cells but not in the cells of the onion root tips, thiamine pyrophosphate is similarly hydrolyzed. Manganese and magnesium ions (1×10^{-3} to $1 \times 10^{-2} M$) stimulate the hydrolysis of both nucleosidediphosphates and thiamine pyrophosphate more than calcium ions do. In many situations, maximum stimulation is given by manganese ions.

A method is outlined for demonstrating the Golgi apparatus in frozen sections of formalin-fixed tissues or smears of cells. It offers a number of advantages over the classical metal impregnation procedures. Most important of these is the cytochemical information it provides. The Golgi apparatus of a variety of cells is illustrated in photomicrographs.

Observations are recorded indicating that high levels of acid phosphatase activity are not localized in the Golgi apparatus of epididymis and most other cells. Rather, they are localized in cytoplasmic granules probably corresponding to lysosomes. Triton X-100, which releases acid phosphatase from the granules presumed to be lysosomes, has no such effect upon the nucleosidediphosphatase activity of the Golgi lamellae.

We wish to thank Miss Phyllis Iaciofano, Miss Barbro Runling, and Mr. Woo-Yung Shin for their valuable technical assistance, and Mr. George Dominguez for assistance with the photography.

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A PARTIAL SEQUENCE OF THE AMINO ACID RESIDUES IN THE γ CHAIN OF HUMAN HEMOGLOBIN F

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That human fetal hemoglobin differs from human adult hemoglobin in its rate of denaturation by alkali was detected almost a century ago by Körber.[1] In the intervening years, other differences (for example, in spectrum, in amino acid composition, etc.) have become apparent. When it was reported[2–4] that the number of N-terminal residues of fetal hemoglobin was only about half that of adult hemoglobin, it was to be expected that the structures of the two molecules would be very different. However, Schroeder and Matsuda[5] showed that fetal hemoglobin has four N-terminal residues of which two are valine and two are glycine; hence, the number of N-terminal residues is the same as in adult hemoglobin, which has four N-terminal valyl residues,[6] and the likelihood of vastly different structures is lessened. Indeed, both fetal[5] and adult[6] hemoglobin have a pair of polypeptide chains that possess the N-terminal sequence val-leu-; available evidence[7–9] suggests that these chains (the α chains) are identical. The other chains (the β chains) of adult hemoglobin are N-terminal in the sequence val-his-leu-,[10] and thus differ from the second kind of chains (the γ chains) of fetal hemoglobin, which are N-terminal in the sequence gly-his-phe-.[9] It is the purpose of this communication to present preliminary data on the sequence of amino acid residues in the γ chain of fetal hemoglobin and to compare these results with sequences in the α and β chains as they have been determined in these laboratories and elsewhere.

General Experimental Procedure.—Umbilical cord hemoglobin from which fetal hemoglobin is ordinarily obtained has been shown by chromatographic procedures[11] to contain not only fetal and adult hemoglobin as has long been known but also other components. In order to have a definite molecular species to study, the main component, termed F_{II}[11], has been isolated chromatographically and used in the present investigation.

Hemoglobin F_{II} was readily isolated in quantity by chromatographing 3 gm of cord hemoglobin on a 3.5 × 35-cm column of ion-exchange resin Amberlite IRC-50 at 6°C with Developer No. 4.[11] When the front of F_{II} reached the bottom of the column, the upper portion that contained the hemoglobin A was removed. By warming the remaining column to 28°C, the F_{II} was eluted from the column in a few hundred ml of developer. It was concentrated for further experiments by high speed centrifugation[12] and then dialyzed free of salt.

The α and γ chains were separated chromatographically by the method of Wilson and Smith[13] as described by them and, in one instance, with the modification of an interrupted gradient as described by Chernoff and Liu.[14]

Enzymatic hydrolyses of individual chains or of heat-denatured hemoglobin F_{II} with trypsin or chymotrypsin were maintained at constant pH by using a Radiometer automatic titrator as a pH-stat. The conditions of hydrolysis were: pH 8.0, 40°C., 0.01 *M* calcium ion, an enzyme: substrate ratio of 1 to 2 per cent by weight, and 90 minutes of hydrolysis with trypsin or 180 minutes with chymotrypsin. After hydrolysis the pH was brought to 6.5 and any insoluble material was removed by centrifuging.

The initial separation of peptides was carried out by column chromatography. The peptides from about 0.5 gm of individual chains or one gm of hemoglobin were chromatographed on a 3.5 × 100-cm column of Dowex 50-X2 ion-exchange resin with a gradient in pH and pyridine concentration The gradient was produced after the principle of the device described by Bock and Ling[15]

in their Figure 6. Three containers of equal diameter were connected in series; in the mixer (the container from which developer went directly to the chromatogram) were placed 4.3 liters of pH 3.1 pyridine-acetic acid buffer that was 0.2 *N* pyridine and in each of the other containers were placed 4.3 liters of pH 5.0 pyridine-acetic acid buffer that was 2 *N* pyridine. A flow-rate of 60 ml per hour was maintained with a Milton Roy Minipump (Milton Roy Co., Philadelphia, Pa.). The ninhydrin procedure[16] was applied to 0.5-ml portions of alternate fractions in order to evaluate the progress of the chromatogram. Fractions which were thus shown to contain peptides were pooled and the solvent was removed largely in a rotary evaporator and finally in a stream of air at 40°C. The homogeneity of the zones thus isolated was investigated by paper chromatography and paper electrophoresis. As necessary, further separation was made by paper chromatography, paper electrophoresis, or column chromatography on Dowex 1.[17]

The amino acid composition of hemoglobin preparations and of purified peptides was determined chromatographically with a Spinco Automatic Amino Acid Analyzer, Model 120. Hydrolysates were prepared by heating in doubly glass-distilled 6 *N* hydrochloric acid at 110°C. for 22 or 24 hours (or for 70 hours also in the case of hydrolysates of hemoglobin) in evacuated sealed tubes. When applicable, hydrolyses were also made with leucine aminopeptidase[18] (LAP) at 40° and pH 8.5 for 16 hours.

The sequence of amino acids in a peptide was determined by the application of a variety of procedures. These procedures included the determination of the N-terminal amino acid by the DNP-method, stepwise degradation by the Edman procedure in a modified form[19] of the paper-strip method, and partial hydrolysis by chemicals or enzymes followed by separation and identification of the fragments.

Results.—Amino acid composition of hemoglobin F_{II} and of the γ chains: The amino acid composition of hemoglobin F_{II} and of the γ chains is presented in Table 1. These data were obtained from two different preparations of hemoglobin F_{II}. A sample from each preparation was hydrolyzed for 22 and 70 hours. The first quantity stated under each time of hydrolysis is from the first sample. The values for serine and threonine after correction for destruction during hydrolysis disagree appreciably. The behavior of cystine is unexpected. If the weight of the heme

TABLE 1

AMINO ACID COMPOSITION OF HEMOGLOBIN F_{II} AND OF THE γ CHAINS

	—Gm Amino Acid per 100 gm Protein—				Residues	Residues				
Amino acid	22-hr. hydrolysis		70-hr. hydrolysis		Residues[a] 66,000 M.W.	$1\alpha + 1\gamma$[b]	α^A chain[c]	γ chain by difference	γ chain[d] from γ_4	γ chain from lit.[e]
Ala	9.09,	8.31	8.78,	8.48	64.2	32	21	11	11.6	13
Arg	3.25,	3.02	3.12,	3.09	11.8	6	3	3	3.3	3
Asp	10.48,	9.73	10.10,	10.01	50.0	25	12	13	12.9	14
Cys/2	0.70,	0.50	0.53,	0.72	3.9	2	1	1	1.1	
Glu	7.99,	7.37	7.68,	7.58	34.3	17	5	12	12.0	12
Gly	4.59,	4.27	4.46,	4.34	38.8	19	7	12	12.2	13
His	8.13,	7.83	7.99,	7.99	34.0	17	10	7	6.7	6
Ileu	1.54,	1.44	1.52,	1.51	7.6	4	0	4	3.7	4
Leu	14.36,	13.35	13.94,	13.79	69.8	35	17–18	17–18	16.3	19–20
Lys	10.15,	10.10	10.04,	10.28	45.8	23	11	12	11.0	12
Met	1.84,	1.73	1.87,	1.74	7.9	4	2	2	1.9	2
Phe	7.74,	7.18	7.46,	7.18	29.6	15	7	8	7.8	8
Pro	4.06,	3.92	4.02,	3.91	22.8	11–12	8	3–4	4.5	4
Ser	6.19,	5.85	4.57,	4.53	43.6 40.6	20–22	11	9–11	9.9	9
Thr	6.71,	6.19	5.77,	5.69	39.6 35.6	18–20	9	9–11	9.5	10
Try							1	4[f]		
Tyr	2.57,	2.75	2.28,	2.41	10.2	5	3	2	1.9	3
Val	9.45,	8.63	9.31,	9.02	51.3	26	13	13	12.6	14

[a] Average of all values except threonine, serine, and tyrosine which are corrected for hydrolytic destruction, and cystine/2 for which only the two highest values are used.
[b] Preceding column divided by 2.
[c] From data of ref. 20.
[d] Values for γ_4 molecule divided by 4 (ref. 21).
[e] Based on ref. 22 by difference.
[f] Tentative estimate from spectrophotometric data

groups and the destruction of serine, threonine, tyrosine, and tryptophan during hydrolysis are taken into account, essentially all of the weight that was taken for analyses is accounted for. Previous analyses of "fetal" hemoglobin[22] have all been performed on material that must have been heterogeneous to some degree. The analyses here presented have been made on preparations from which the other obvious components of cord blood hemoglobin have been removed. The results of the present analyses in general are in good agreement with the average of the sometimes disparate quantities previously reported.[22] If it is assumed that the α^A and α^F chains are identical, the amino acid composition of the γ chain can be calculated. An amino acid analysis of the separated γ chains has not been made because N-terminal analyses suggest that our best preparations contain 15 to 25 per cent of α chains.

Initial chromatographic separation of tryptic and chymotryptic peptides: The chromatographic separation of the soluble tryptic peptides in a hydrolysate of hemoglobin F_{II} is depicted in Figure 1, in which the optical density after reaction

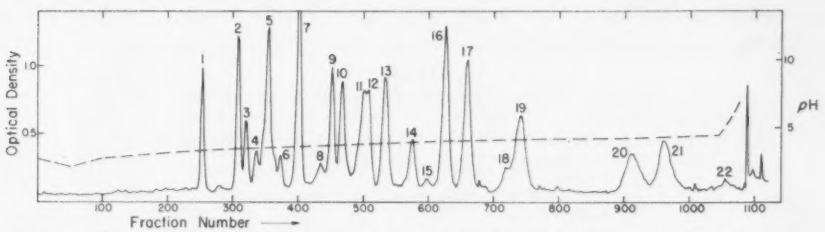

FIG. 1.—Separation of peptides from a tryptic hydrolysate of hemoglobin F_{II} by chromatography on a column of Dowex 50-X2 with pyridine-acetic acid developers. At Fraction No. 985, elution was begun with 2 *N* sodium hydroxide.

with ninhydrin is plotted against the fraction number of the 10-ml fractions. The pH gradient is also shown. The peptides in the peaks of this chromatogram are derived from both the α and the γ chains, but the chain of origin is known from similar chromatograms of hydrolysates of the individual chains. In this chromatogram, the peaks that are numbered 1, 2, 3, 5, 9, 10, 11, 12, 13, 16, 18, 19, and 21 contain peptides that derive from the γ chain, and those numbered 2, 3, 5, 6, 7, 11, 13, 14, 16, 17, 19, 20, and 21 contain peptides from the α chain.

The chromatographic isolation of the peptides in a chymotryptic hydrolysate of γ chains is presented in Figure 2. The chromatographic conditions were identical in the chromatograms that are shown in Figures 1 and 2, but the amount of sample was different. The ninhydrin reaction was used only roughly quantitatively to detect the zones; comparison of the sizes of the peaks is indicative of the relative amount only in a given chromatogram.

The peptides from both hydrolysates were separated and purified by paper chromatography, paper electrophoresis, or column chromatography as necessary.

Sequence of the tryptic peptides of the γ chain: After N-terminal determination and amino acid analysis had demonstrated the purity of a given peptide, the sequence was determined by the application of such methods as were suggested by the amino acid composition. The sequence of amino acid residues in the soluble tryptic peptides of the γ chain is presented in Table 2.

It is known that the γ chain is N-terminal in the sequence gly-his-phe-.[9] The

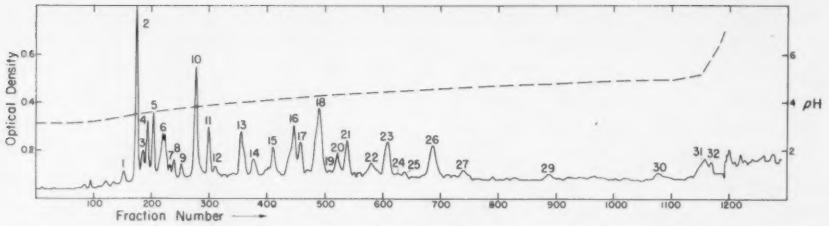

FIG. 2.—Separation of peptides from a chymotryptic hydrolysate of γ chains by chromatography on a column of Dowex 50-X2 with pyridine-acetic acid developers. At Fraction No. 1098, elution was begun with 2 *N* sodium hydroxide.

sole peptide that contains this sequence is that from Zone 19. In this peptide, which contains two lysyl residues, the bond between lysine and alanine apparently is hydrolyzed with difficulty. The N-terminal 8-residue portion has not been isolated from the hydrolysate and the C-terminal 9-residue portion has been isolated only in very small amount from Zone 13. The peptides in Zones 13 and 19 contain one residue each of leucine and isoleucine. It is still undetermined, however, which one occupies the indicated positions.

TABLE 2

AMINO ACID SEQUENCE IN SOLUBLE TRYPTIC PEPTIDES OF THE γ CHAIN

Zone no.	Peptide
1	val-aspNH_2-val-glu-asp-ala-gly-gly-glu-thr-leu-gly-arg
2	val-leu-thr-ser-leu-gly-asp-ala-ileu-lys
3	lysine
5	met-val-thr-gly-val-(ala,ser,ala,leu,ser,ser)arg
9	val-lys
10	his-leu-asp-asp-leu-lys
11	lys-(asp,thr,ser,gly,ala,val,ileu,leu_2)lys
12	tyr-his
	leu ileu
13	ala-thr- or -thr-ser- or -try-gly-lys
	ileu leu
16	ala-his-gly-lys
18	leu-leu-val-val-tyr-pro-try-(thr,glu)-arg
	leu ileu
19	gly-his-phe-thr-glu-glu-asp-lys-ala-thr- or -thr-ser- or -try-gly-lys
	ileu leu
21	ala-his-gly-lys-lys

The amino acid composition of the peptide in Zone 1 differs only slightly from a peptide of the β chains: in the γ chain peptide, one threonyl residue replaces a valyl residue. Hunt and Ingram[23] in a study of hemoglobin E have determined the sequence of the β chain peptide. Although the β and γ chain peptides differ in gross composition by a single amino acid residue, they differ in four ways in sequence, as the following comparison shows:

β chain: val-aspNH_2-val-asp-glu-val-gly-gly-glu-ala-leu-gly-arg

γ chain: val-aspNH_2-val-*glu-asp-ala*-gly-gly-glu-*thr*-leu-gly-arg.

The position of the amide group in both peptides was determined in the course of the present investigation.

From a chymotryptic hydrolysate of the γ chains (Zone 10, Figure 2), the peptide gly-lys-val-aspNH_2-val-glu(asp,ala,gly,gly,glu,thr,leu) has been isolated. This peptide establishes the connection between the N-terminal peptide in Zone 19 and the peptide in Zone 1.

The peptide in Zone 18 has been isolated only in rather small amount. How-

ever, it probably follows the peptide of Zone 1 in the sequence of the γ chains. The conclusion is derived from the isolation of gly-arg-leu-leu from Zone 23 of the chymotryptic hydrolysate.

These three tryptic peptides in the order 19, 1, and 18 provide the sequence of the first 40 residues from the N-terminus of the γ chain. The remaining tryptic peptides in Table 2 account for 37 additional residues whose placement in the chain can be inferred only indirectly at present. Because the amino acid analysis of γ chains as presented in Table 1 suggests a content of about 145 residues, the soluble tryptic peptides account for little more than half of the chain. Amino acid analysis of that portion of the γ chain which is insoluble at pH 6.5 after tryptic hydrolysis (the so-called "core") is in general agreement with the number and kind of residues as yet unaccounted for. The position of the remaining soluble tryptic peptides in the sequence of the γ chain is best considered in relation to information that is now available about the β chain.

Table 3 presents the sequence of soluble tryptic peptides of the β chain. The

TABLE 3

AMINO ACID SEQUENCE IN SOLUBLE TRYPTIC PEPTIDES OF THE β CHAIN

Zone no.	Peptide
1	val-aspNH_2-val-asp-glu-val-gly-gly-glu-ala-leu-gly-arg
2 + 3	phe-phe-glu-ser-phe-gly-asp-leu-ser-(ala,val,pro_2,asp_2,thr,met,gly)-lys
3	glu-phe-thr-pro-(tyr,val,ala_2,pro,glu_2)lys
4	lysine
8	val-val-ala-gly-val-ala-aspNH_2-ala-leu-ala-his-lys
9	val-lys
9	val-leu-gly-ala-phe-ser-asp-gly-leu-ala-(his,asp_2,leu_2)-lys
10	val-his-leu-thr-pro-glu-glu-lys
12	tyr-his
13	ser-ala-val-thr-ala-(leu,try,gly)-lys
15	val-leu-gly-ala-phe(ser,asp,gly,leu,ala,his,asp_2,leu_2)lys_2
16	ala-his-gly-lys
18	leu-(leu,val_2,pro,thr,gly,tyr,try)arg
20	val-val-(ala_4,asp,gly,his,leu,val,lys,tyr,his)
22	ala-his-gly-lys-lys

peptides were isolated by the methods that were applied also to the γ chain. The numbering of the zones coincides roughly with that of the zones in Figure 1.

Braunitzer and co-workers[24] have presented a partial formula of the β chain and have stressed[25] the homology between the α and β chains. The N-terminal sequences of the β and γ chains may be compared as follows:

```
                                                  10
β- val-his-leu-thr-pro-glu-glu-lys-ser-ala- val-thr-ala-
                                                    leu
γ- gly-his-phe-thr-glu-glu-asp-lys-ala-thr- or -thr-ser-
                                                    ileu
                                  20
β- -leu-try-gly-lys-val-aspNH2-val-asp-glu-val-gly-gly-glu-
   ileu
γ- - or -try-gly-lys-val-aspNH2-val-glu-asp-ala-gly-gly-glu-
   leu
                30                                      40
β- -ala-leu-gly-arg-leu-leu(thr,glu,pro,val,val,try,tyr)-arg-phe-etc.
γ- -thr-leu-gly-arg-leu-leu-val-val-tyr-pro-try(thr,glu)-arg-
```

The general homology of the two chains is apparent. Although the basic amino acids fall in identical positions, the other residues are subject to considerable substitution and rearrangement. Nevertheless, other peptides of the γ chain may be compared to those of the β chain on this basis. Clearly, val-lys, tyr-his, and ala-his-gly-lys-lys are present in the hydrolysate of both chains. The decapeptide of Zone 3 of the γ chains is N-terminal in val-leu- as is the hexadecapeptide of Zone 9 of the β chains, but other identity extends only to aspartic acid in the seventh position from the N-terminus. On the other hand, the C-terminal sequence of the β peptide is[24] -his-leu-asp-asp-leu-lys, a sequence identical with that of the peptide in Zone 10 of the γ chain chromatogram. One is tempted to suggest that the γ chain peptides in Zones 3 and 10 are adjacent in the chain. Braunitzer et al.[24] report a sequence from the 56th through the 85th residue of the β chain which may be fitted with sequences from the γ chain as follows:

	60	70
β-	-gly-asp-------pro-lys-val-lys-ala-his-gly-lys-lys-val-leu-gly-ala-phe-ser-	
γ-	-gly-aspNH_2-pro-lys-val-lys-ala-his-gly-lys-lys-val-leu-thr-ser-leu-gly-	
	80	
β-	-asp-gly-leu--ala-his-leu-asp-asp-leu-lys-gly-thr-phe-	
γ-	-asp-ala-ileu-lys-his-leu-asp-asp-leu-lys-gly-thr-phe-.	

Although their structures are incompletely determined, the isolation of certain peptides from the γ chain lends credence to this arrangement. The peptides gly-aspNH_2-(pro,val,lys_2) and gly(val,leu,lys_2) from the chymotryptic hydrolysate suggest the sequences from the 56th to the 61st, and from the 64th to the 68th residues. The link between the 66th and 67th also follows from the tryptic peptide of Zone 11. It differs in amino acid composition from that of Zone 2 (67th to 76th residues) only in the presence of an additional lysine which is known to be N-terminal. The final three residues of the β sequence are derived from the core: this same sequence may be isolated from a chymotryptic digest of the γ core and, in addition, the sequence lys-gly-thr-phe from a chymotryptic hydrolysate of the γ chains. (*Note added in proof:* More recently isolated peptides confirm the sequence suggested here for these 30 residues of the γ chain.)

The sequence tyr-his no doubt is derived from the C-terminus of the γ chain. The work of Naughton[26] suggests this as well as the fact that free histidine may be detected in a chymotryptic hydrolysate of the γ chain (Zone 11, Fig. 2). Tyr-his is also C-terminal in the β chain.[24]

There is a meager resemblance in the γ-chain peptide of Zone 6 and the β-chain peptide of Zone 8 as follows:

β val-val-ala-gly-val-ala-aspNH_2-ala-leu-ala-his-lys
γ met-val-thr-gly-val-(ala,ser,ala,leu,ser,ser)-arg.

The sequence within the parentheses is probable although not yet confirmed. A β chain peptide of this composition plus tyr-his make up the sequence of the last 14 residues from the C-terminus of the β chain according to Braunitzer *et al.*[24] Perhaps this γ chain peptide plus tyr-his also is C-terminal in the γ chain. It is of interest to note that our sequence for this β chain peptide differs much from that of Braunitzer *et al.*, who report it as val-val-val-ala-ala-gly-ala-asp-leu-ala-his-lys.

If the above conclusions are correct and the suggested homology exists between

the β and γ chains, then the sequences in the γ chain are known between residues 1 to 40, approximately 56 to 85, and approximately 137 to 150 and unknown sequences exist between residues 41 to 55 and 86 to 136. In the β chain, residues 41 to 59 are taken by the peptide in Zones 2 and 3 (Table 3) but no similar peptide has yet been detected in the soluble tryptic peptides of the γ chain. Presumably, it is present in the insoluble fraction. The presence of such a peptide in positions 41 to 59 of the γ chain is suggested by the isolation of the peptide gly-aspNH_2(pro, val,lys_2) from a chymotryptic hydrolysate which, as noted above, may coincide with positions 56 to 61 of the β chain. Certain sequences of yet undetermined position are known through the isolation of peptides from a chymotryptic hydrolysate of the γ core or the γ chain: these sequences are asp-ser-phe, ala-ser-try, ala-ileu-his-phe, and ala-gluNH_2-leu-ser-glu-leu.

Sequences in the α chains of hemoglobin A and F: The investigation of the γ chains also provides opportunity for the isolation of the α chain of hemoglobin F and of the peptides derived therefrom by tryptic hydrolysis. One can, therefore, begin to determine on a far more exact basis whether the α chains of hemoglobins A and F are identical. The tryptic peptides of the α chains of hemoglobin A are listed in Table 4. It is of interest that sequences in peptides of Zones 11 and 13

TABLE 4

AMINO ACID SEQUENCE IN SOLUBLE TRYPTIC PEPTIDES OF THE α^A CHAIN

Zone no.	Peptide
2	val-leu-ser-pro-ala-asp-lys
3	val-asp-pro-val-aspNH_2-phe-lys
3	lys
5	val-ala-asp-ala-(his,thr,ser,ala,val,leu)(ala_4,asp_2,val,leu_2,ser,pro,his_2,met)-lys
6	thr-aspNH_2-val-lys
7	val-leu-ser-pro-ala-asp-lys-thr-aspNH_2-val-lys
11	met-phe-leu-ser-phe-pro-thr-thr-lys
13	val-gly-ala-(his,ala,gly,glu)tyr-gly-ala-glu-ala-leu-glu-arg
14	leu-arg
16	gly-his-gly-lys
17	ala-ala-try-gly-lys
19	tyr-arg
20	thr-tyr-phe-pro-his-phe-asp-leu-ser-ser-gly-ala-(gluNH_2,his,val)-lys
21	gly-his-gly-lys-lys

are at variance with those reported by Braunitzer *et al.*[20] In the 6th to 8th positions of the peptide in Zone 11, they give the sequence as -thr-pro-thr- instead of -pro-thr-thr-; Hill and Konigsberg[27] agree with our sequence. In the 9th to 12th positions of the peptide in Zone 13, Braunitzer *et al.* give the sequence -gly-glu-ala-ala- instead of -gly-ala-glu-ala.

Only a limited investigation of the peptides from the α^F chains has yet been made. In particular, the peptides in Zones 7, 13, 17, and 21 have been studied. The amino acid composition is identical with that of similar peptides of α^A chains. The sequence is identical in the peptide from Zone 17 and also in the other peptides to the extent that it has been studied. These investigations are continuing.

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THERMODYNAMIC POTENTIALS OF SYMMETRICAL ELECTROLYTES

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The classical treatment of the thermodynamic properties of electrolytic solutions by Debye and Hückel[1] leads to the now familiar limiting square-root law which has been amply confirmed by experiment. At nonzero concentrations, however, positive deviations from the limiting law appear; there has been considerable speculation about their physical origin since 1923. The purpose of this communication is to show, by means of a more detailed study of the ionic potentials, that the properties of electrolytic solutions, specific for each electrolyte at nonzero concentrations, do not appear to be derivable by extension of the limiting solution of the Poisson-Boltzmann equation to higher concentrations, but rather must be sought in the projection of a theory of fused salts into and through the region of concentrated solutions.

We shall use the conventional model of charged spheres of diameter a in a dielectric continuum to represent the solution. Let there be n_i ions of charge ϵ_i in a

total volume V. Restriction to the case of simple electrolytes then requires that

$$n_1\epsilon_1 + n_2\epsilon_2 = 0 \tag{1}$$

where $n_1 = n_2 = n$ and $\epsilon_1 = \epsilon = -\epsilon_2$, with ϵ equal to the elementary charge times the valence. The Poisson-Boltzmann equation for the potential ψ_j of an ion of species j then takes the form

$$\Delta\psi_j = (\kappa^2\epsilon/D\beta) \sinh (\epsilon\psi_j/kT), \tag{2}$$

where

$$\kappa^2 = 8\pi n\epsilon^2/DkT \text{ cm}^{-2} \tag{3}$$

and

$$\beta = \epsilon^2/DkT \text{ cm} \tag{4}$$

By expansion of the hyperbolic sine as a series and neglect of all but the leading term, (2) is linearized and has the well-known solution

$$\psi_j = \epsilon_j e^{-\kappa r} e^{\kappa a}/rD(1 + \kappa a) \tag{5}$$

when the boundary conditions $\psi_j(\infty) = 0$ and continuity of potential and field strength at $r = a$ are satisfied.

Reduction of (2) to a linear equation implies that $\epsilon\psi/kT$ may be considered so small compared to unity that the cubic and higher terms in the series expansion may be neglected. For hypothetical solvents of very high dielectric constant and for distances $r \gg a$ in general, this approximation is justified, but *at ionic contact*, even in water ($D = 78.54$), the procedure appears hazardous: in this case, $\epsilon\psi/kT \approx \epsilon^2/aDkT = 2$ for $a = 3.57 \times 10^{-8}$ cm and $\sinh 2 = 3.63$.

It has been clearly established that the screening term $e^{-\kappa r}$ correctly accounts for the long-range interactions of ions, but the high-energy short-range electrostatic interactions have been too casually rejected as negligible fluctuation terms. Bjerrum,[2] by his *ad hoc* hypothesis of ion association, made the most successful approach to the problem of dealing with the effects of pairwise contacts of ions of opposite charge, and a substantial body of experimental observations has justified the fundamental soundness of explicitly including these effects in a general description of the properties of electrolytic solutions. Our treatment of the problem is in effect a re-integration of the Poisson-Boltzmann equation, using boundary conditions which permit the reference ion to control the electrostatic situation in its immediate vicinity, while still allowing for the atmosphere screening at large distances.

The distinction between "large" and "small" distances will be made on the basis of a criterion originally proposed by Bjerrum. It will be recalled that the probability that an anion and a cation will be found at a distance r from each other has in general[3–6] a minimum near the distance $r = \beta/2$. For short distances, where, by hypothesis, $r \leq \beta/2$, we set

$$\psi_S = \epsilon/Dr + \varphi(r) \tag{6}$$

where ϵ/Dr is the potential at a distance r from an isolated ion and $\varphi(r)$ is the perturbation in the Coulomb potential produced by nearby ions in a real solution. (Even in dilute solutions, short-range approaches will occasionally occur.) Substitution in (2) gives

$$\Delta\varphi = (\kappa^2\epsilon/2D\beta)e^{\beta/r}e^{\epsilon\varphi/kT}[1 - \exp\{-(2\beta/r + 2\epsilon\varphi/kT)\}]. \tag{7}$$

The exponential term in the brackets is in any practical case negligible compared to unity; as a first approximation, we shall also neglect $\epsilon\varphi/kT$ compared to β/r in the exponent of the coefficient (*i.e.*, approximate $e^{\epsilon\varphi/kT}$ by unity), thereby reducing (7) to the equation

$$\Delta\varphi = (\kappa^2\epsilon/2D\beta)e^{\beta/r}. \tag{8}$$

Integration and substitution in (6) gives

$$\psi_S = \epsilon/Dr + (\kappa^2\beta\epsilon/2D)[A\beta/r + B + F_1(r)], \tag{9}$$

where A and B are coefficients of the solutions of the homogeneous equation and $F_1(r)$ is the particular integral of (8):

$$F_1(r) = e^{\beta/r}(r^2/6\beta^2 + r/3\beta - 1/6) + (\beta/6r - 1/2)\mathrm{Ei}(\beta/r). \tag{10}$$

In (10), $\mathrm{Ei}(x)$ is the positive exponential integral

$$\mathrm{Ei}(x) = \int_{-\infty}^{x} (e^u/u)du. \tag{11}$$

For distances $r \geq \beta/2$, the long-range potential is obtained by integrating the appropriate approximation of (2):

$$\psi_L = (\kappa^2\epsilon/D\beta)[\epsilon\psi/kT + 1/6(\epsilon\psi/kT)^3], \tag{12}$$

which has the solution

$$\psi_L = Ce^{-\kappa r}/r + (\kappa^2\epsilon\beta/2D)(2\beta e^{\kappa r}E_4/3r - \beta e^{-\kappa r}E_2/3r) \tag{13}$$

where E_n is the negative exponential integral

$$E_n = \overline{\mathrm{Ei}}(n\kappa r) = \int_{n\kappa r}^{\infty} (e^{-u}u/)du. \tag{14}$$

(In (13), we have of course dropped the homogeneous solution $e^{\kappa r}/r$ in order to satisfy the boundary condition $\psi_L(\infty) = 0$.)

We now have (9) for the potential near the reference ion and (13) for the potential at distances beyond $\beta/2$; the integration constants must next be evaluated. The first can be determined from the condition of electroneutrality

$$-\epsilon = \int_a^{\infty} 4\pi\rho r^2 dr; \tag{15}$$

that is, the integrated space charge must exactly balance the charge on the reference ion. The other two constants can be evaluated by using the fact that potential and field strength are continuous for $a \leq r \leq \infty$ and specifically at $r = \beta/2$:

$$\psi_S(\beta/2 - 0) = \psi_L(\beta/2 + 0) \tag{16}$$

and

$$(d\psi_S/dr)_{\beta/2-0} = (d\psi_L/dr)_{\beta/2+0}. \tag{17}$$

Substitution of the Poisson relation between potential and density in (15) gives

$$\epsilon = D\int_a^{\infty} r^2\Delta\psi dr, \tag{18}$$

and integration, with the Laplacian operator in spherical coordinates, immediately gives

$$\epsilon = D\left[r^2\frac{d\psi_s}{dr}\right]_a^{\beta/2} + D\left[r^2\frac{d\psi_L}{dr}\right]_{\beta/2}^{\infty}, \tag{19}$$

whence, using (17),

$$(d\psi_S/dr)_a = -\epsilon/Da^2. \tag{20}$$

Differentiation of (9) and substitution of (20) in the result evaluates A:

$$bA = (e^b/6b^2)(2 + b + b^2) - (b/6)\mathrm{Ei}(b), \tag{21}$$

where b is the Bjerrum parameter

$$b = \epsilon^2/aDkT. \tag{22}$$

Elimination of C from (16) and (17) and substitution of

$$A = F_2(b)/b \tag{23}$$

from (21) evaluates B:

$$B = (1 + \tau)^{-1}[-1/\tau - G_3(\tau) - F_2(2) - (1 + \tau)F_1(2) - 2\tau F_2(b)/b], \tag{24}$$

where $$G_3(\tau) = 2e^{-3\tau}/3 - 8\tau e^{\tau}\overline{\mathrm{Ei}}(4\tau)/3 \tag{25}$$

and $$\tau = \beta\kappa/2 = 4.2016 \times 10^6 c^{1/2}/(DT)^{3/2}. \tag{26}$$

In order to calculate the electrostatic contribution G_e to the free energy, we first isolate the potential ψ^* at the reference ion due to the presence of the other ions, where

$$\psi^* = \psi_S(a) - \epsilon/Da. \tag{27}$$

Substitution of $r = a$ and the values given by (23) and (24) for A and B in (9) yields

$$\psi^* = [\kappa^2\beta\epsilon/2D(1 + \tau)][-1/\tau - G_3(\tau) - H(b,2) - \tau L(b,2)] \tag{28}$$

$$= [\kappa^2\beta\epsilon/2D(1 + \tau)][-1/\tau - f(b,\tau)], \tag{29}$$

where $$H(b,2) = {}^1/_2\mathrm{Ei}(b) - {}^1/_2\mathrm{Ei}(2) - e^b(1 + b)/2b^2 + 3e^2/8 \tag{30}$$

and $$L(b,2) = {}^1/_6\mathrm{Ei}(b) - {}^1/_6\mathrm{Ei}(2) - e^b(b^2 + b - 4)/6b^3 + e^2/24. \tag{31}$$

Then the relations

$$G_e = {}^1/_2\textstyle\sum_j n_j\epsilon_j\psi_j^* \tag{32}$$

and $$kT \ln f_j = \partial G_e/\partial n_j \tag{33}$$

give for the activity coefficient

$$-\ln f_j = \tau/(1 + \tau) + [\tau^2/(1 + \tau)][G_3(\tau) + H(b,2) + \tau L(b,2)]. \tag{34}$$

While the leading term of (33) is independent of the charging process used to derive G_e, the detailed form of the higher terms will depend on the process chosen[7]; these details are not pertinent to the present discussion.

Let us first examine the limiting form of (34) for very low concentrations, where terms of order τ^2 (proportional to concentration c) in the second term of (34) may be disregarded:

$$-\ln f_j = \tau/(1 + \tau). \tag{35}$$

This equation states that the limiting law for the activity coefficient for ions of finite size is *independent of ion size;* the corresponding Debye-Hückel relation is

$$-\ln f_j = \tau/(1 + \kappa a). \tag{36}$$

A comparison of the two results with experiment is shown in Figures 1 and 2, where

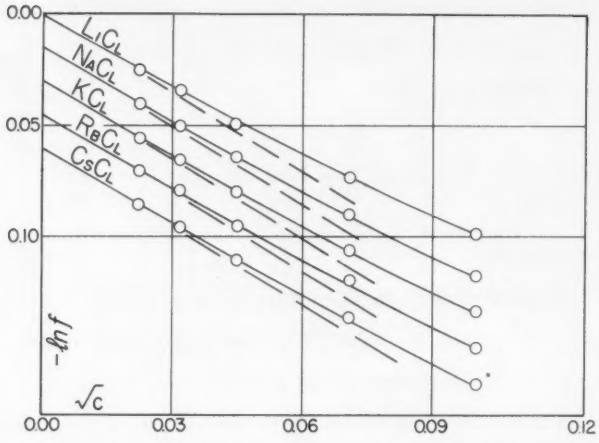

FIG. 1.—Comparison of experimental results with (36).

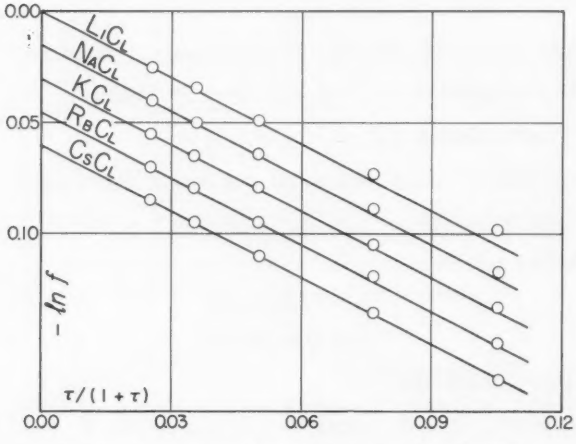

FIG. 2.—Comparison of experimental results with (35).

data[8] for the alkali halides in water are plotted; in Figure 1, $(-\ln f)$ is plotted against square root of concentration and in Figure 2 against $\tau/(1 + \tau)$ where $\tau = 1.1725c^{1/2}$ for water at 25°. (Successive plots for the various halides are displaced vertically by constant amounts to avoid confusion.) It will be seen that the experimental values begin to show positive deviations from the limiting tangents (dashed lines of Fig. 1) at quite low concentrations; by inclusion of the term in κa, the deviations can, of course, be reduced, but at the price of using an arbitrary constant a. Equation (35), on the other hand, not only predicts the limiting tangent, but gives the first-order deviations. The difference between (35) and (36) is simply the appearance of the Bjerrum radius $\beta/2$ in the denominator of (35) in place of the ionic contact distance a in (36). Functions of this form for the activity

coefficient have also been proposed by Grunwald[9] and by Kortüm[10] on the basis of heuristic arguments; the present derivation shows that (34) is a direct consequence of the Poisson-Boltzmann equation alone, plus the continuity and neutrality conditions, without an added hypothesis regarding ion pair formation.

We next consider the higher terms[11] of (34). These are of two kinds: those depending only on τ and those depending on both a and τ. We note first that the former further *decrease* ln f. We have carried out the integration of (2), retaining the next highest term in the series. The corresponding contribution G_5 to ln f likewise decreases this quantity; furthermore, G_5 is considerably smaller than G_3 as shown in the accompanying table of numerical values.

TABLE 1

VALUES OF $G_3(\tau)$ AND $G_5(\tau)$

τ	0.0	0.2	0.4	0.6	0.8	1.0
G_3	0.6667	0.2454	0.0952	0.0409	0.0186	0.0087
G_5	0.0444	0.0122	0.0074	0.0062	0.0047	0.0031

These terms represent volume effects in the sense that they arise from the fields of ions distant from the reference ion at the origin. The experimental observation is that activity coefficients usually go through a minimum with increasing concentration, eventually exceeding unity. The above analysis strongly suggests that the observed positive deviations from the limiting law cannot find their origin in the inclusion of further terms of the Poisson-Boltzmann equation, which, as we have just seen, work in the opposite direction. Furthermore, the positive deviations are characteristic of a given electrolyte, and one cannot expect long-range volume effects to produce specific differences from electrolyte to electrolyte. We therefore must conclude that the observed positive deviations are due to persistence into the moderately dilute range of concentrations of the distribution functions which characterize the fused salt and which obviously must depend on size and geometry of the ions in a highly specific way. As we remarked in the introduction, we therefore believe that a final theory of electrolytic solutions must proceed from a theory of the fused salt.

Next, we consider the other higher terms of (34), represented by the functions $H(b,2)$ and $L(b,2)$. The second is of order $c^{3/2}$ and may be disregarded for present purposes. The first takes the suggestive form

$$\tau^2 H/(1 + \tau) \approx 6\pi N a^3 e^b c/3000 \tag{37}$$

for large values of b. This term in the thermodynamic potential has its origin in configurations involving pairs of ions in contact, *via* the explicit appearance of a, and is precisely the expression which would have appeared if we had used the Debye-Hückel limiting law for the "free" ions and then grafted on, as did Bjerrum, the *ad hoc* hypothesis that short-range contacts can be accounted for by considering the ions involved as "associated" to pairs and not contributing to the atmosphere of any ion. Furthermore, the coefficient of c in the asymptotic expansion (37) is, within a numerical factor of $^2/_3$, the value calculated by Fuoss[12] for the association constant K_A. We thus see that the effects which have been ascribed to ion pairs are simply the expected consequences of a more rigorous integration of the Poisson-Boltzmann equation in which these contacts are explicitly included.

One significant practical difference between (35) and (36) calls for comment. If (35) is accepted, then clearly "ion sizes" computed from (36) plus an added empirical term Bc lose all physical meaning whatsoever, and we must consider new empirical interpolation formulas for the representation of activity coefficients at finite concentrations. Since theory so far offers no help in suggesting the form of the function, any convenient functions, such as $c \log c$ or $c^{1/3}$ may be used, *provided* they are introduced in such a way that they do not conflict with the limiting square-root law, $\ln f \sim \tau$.

We have also reconsidered the conductance problem from the present point of view. Briefly summarized, the results are as follows: (1) a universal reduced conductance equation in terms of the rational variable τ and a hydrodynamic constant proportional to viscosity is obtained; (2) in addition to the decrease in mobility due to volume effects (electrophoresis, and relaxation and velocity fields), a further decrease in conductance appears which is the explicit consequence of ions approaching to contact. The functional form of the latter has exactly the form which would appear as a consequence of hypothesizing ionic association; moreover, the coefficient contains the expected exponential term e^b. These results are being prepared for publication.

The description of electrolytic properties in terms of a single universal dimensionless variable τ for all simple electrolytes appears to be fundamentally significant. This variable is the ratio of two distances, $\beta/2$ which in effect states the limit up to which the central ion has control, and $1/\kappa$ which is the parameter characteristic of the long-range screening of the reference ion by the ionic atmosphere. Dimensional analysis shows that τ should indeed be the rational independent variable for the description of electrolytic solutions: we are dealing with electrostatic interactions between ions at average distances proportional to $V^{1/3}$ with corresponding energies $\epsilon^2 c^{1/3}/D$. The order which the electrostatic forces attempt to maintain is opposed by Brownian motion; the ratio $\epsilon^2 c^{1/3}/DkT$ therefore logically appears; we would expect the description of the system to be in the form $f(c^{1/3}/DT)$, and indeed τ is proportional to $(c^{1/3}/DT)^{3/2}$. There thus appears to be some element of justification for the arguments that the cube root of concentration should appear in the theory of electrolytes, and there are in fact many reasons to believe that at moderate and high concentrations, electrolytic properties do become simple functions of $c^{1/3}$. As Debye pointed out,[1] the fallacy in the Ghosh[13] model was the disregard of kT and the consequent attempt to extrapolate to zero concentration on a $c^{1/3}$ scale. In the fused salt and in highly concentrated solutions, however, interionic distances are necessarily of the order of $c^{-1/3}$; here of course the dielectric constant presents some serious problems. But it seems reasonable to predict that the goal of electrolyte theory will be the elucidation of the functions in expressions of the form

$$P = f_1(\tau)(1 - g) + f_2(\tau)g(V_S/V) \tag{38}$$

where P is a property of the solution, V_S is the volume of solute in a total volume V, and g is a partition function, possibly of the form proposed by Eyring,[14] such that $g(1) = 1$ and $g(0) = 0$. Our present thesis is that the limiting form of f_1 is given by (35) when P is the thermodynamic potential and by other explicit functions of τ when P describes one of the irreversible processes such as conductance or diffusion.

* Contribution No. 1660.

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THE NET HYDRATION OF DEOXYRIBONUCLEIC ACID,†*

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In 1954, Jacobson *et al.*[1] presented evidence for the extensive hydration of DNA from studies of the proton magnetic resonance in aqueous solutions of sodium DNA. The following year Wang[2] concluded that DNA was hydrated to the extent of only 0.35 gm water/gm dry deoxynucleate from self-diffusion measurements of water in NaDNA solutions. This paper presents evidence for a net hydration of 0.2 to 2 gm water/gm CsDNA in certain buoyant solvents.

The hydration of T-4 bacteriophage DNA[3] has been studied in density gradient systems at sedimentation equilibrium in the ultracentrifuge.[5] Williams *et al.*[6] showed that the buoyant density is that of the solvated species. The buoyant density ρ_0 is defined by the following thermodynamic equations:

$$\frac{1}{\rho_0} = \frac{M_3\bar{v}_3 + \Gamma M_1\bar{v}_1}{M_3 + \Gamma M_1},$$

where 1 refers to water, 3 to the unhydrated polymer, and

$$\Gamma = -\left(\frac{\partial\mu_1}{\partial m_3}\right)_{T,P,m_1} \Bigg/ \left(\frac{\partial\mu_1}{\partial m_1}\right)_{T,P,m_3} = \left(\frac{\partial m_1}{\partial m_3}\right)_{T,P,\mu_1}.$$

M, $\bar{v}$, μ, and m are molecular weights, partial specific volumes, chemical potentials, and molalities respectively. Molalities for this equation are expressed in moles per unit weight of salt. The unusual definition of molality is necessary so that Γ, the net solvation, remains a positive quantity.[7]

The net solvation of DNA is shown here to be a monotonic function of water

activity. Introducing the concept of water activity into the problem of polymer hydration has proven useful.

The first data were obtained on solution mixtures of CsBr and LiBr. On addition of small amounts of LiBr to CsBr solution there is a sudden drop in the buoyant density associated with the replacement of Cs ions on the DNA by Li ions (Fig. 1).

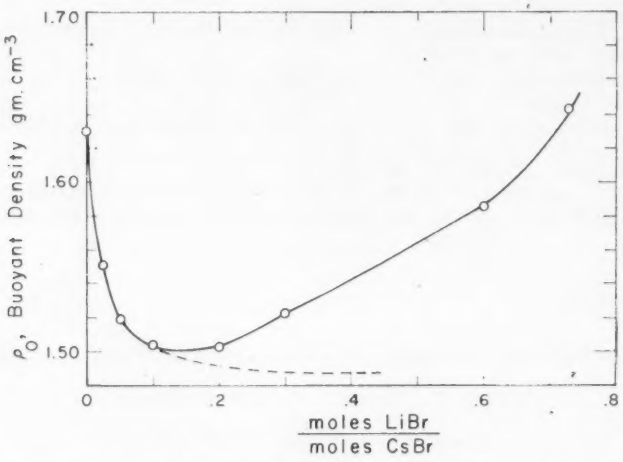

FIG. 1.—Buoyant density of T-4 bacteriophage DNA in aqueous mixtures of LiBr and CsBr at 25°C.

In the event that solvation of the DNA does not change, it can be shown that the plot of ρ_0 against the mole ratio Li/Cs in the solution should be approximately hyperbolic. The limiting value of ρ_0 at Li/Cs = ∞ is the buoyant density of the solvated lithium DNA. Curves of this form have been obtained for mixtures of CsBr and guanidinium bromide and for CsCl and guanidinium chloride.[8]

Although the curve for LiBr-CsBr mixtures is hyperbolic at low LiBr concentration, the buoyant density increases at high LiBr concentrations. At these LiBr concentrations the DNA is entirely in the lithium form as indicated in Figure 1 by the dashed hyperbola. The increase in buoyant density of the solvated LiDNA is then to be expected, as solvation should decrease at the low-water activities in concentrated lithium bromide solutions.

The same data with additional points have been plotted against water activity in Figure 2. The water activities were calculated with the Guggenheim rule for mixed electrolytes and with data for osmotic coefficients tabulated by Robinson and Stokes.[9] Because osmotic coefficients for CsBr are not available at high mixed salt molalities, the osmotic coefficients of CsCl were used for CsBr for total molalities between 5 and 10. Above molality 10 the osmotic coefficient of CsBr was taken to be 1.02. Errors resulting from this procedure are small as the mole fraction of CsBr is small. The buoyant density in aqueous LiBr was not obtained because the salt is not soluble enough at 25°C.

A second method demonstrating the effects of water activity on buoyant density is shown in Figure 3. The buoyant densities[10] of T-4 DNA in different cesium

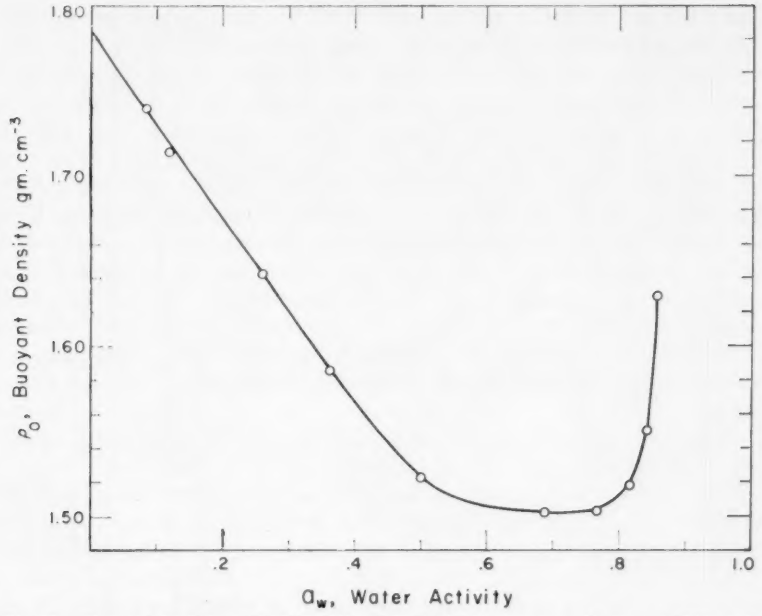

FIG. 2.—Buoyant density of T-4 bacteriophage DNA in aqueous mixtures of LiBr and CsBr at 25°C.

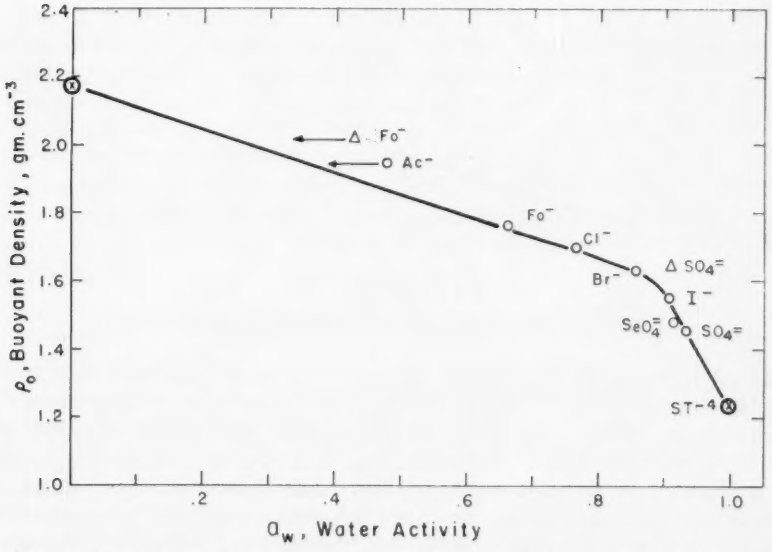

FIG. 3.—Buoyant density in various aqueous cesium salt solutions. T-4 bacteriophage DNA, ○; Pea seedling ribosomal RNA, △, in $SO_4^=$; *Escherichia coli* ribosomal RNA, △ in Fo^-. The extreme DNA values, ⊗, were obtained with LiDNA and recalculated for CsDNA as described in the text. Fo^-, Ac^-, St^{-4} refer to the formate, acetate, and silicotungstate ions.

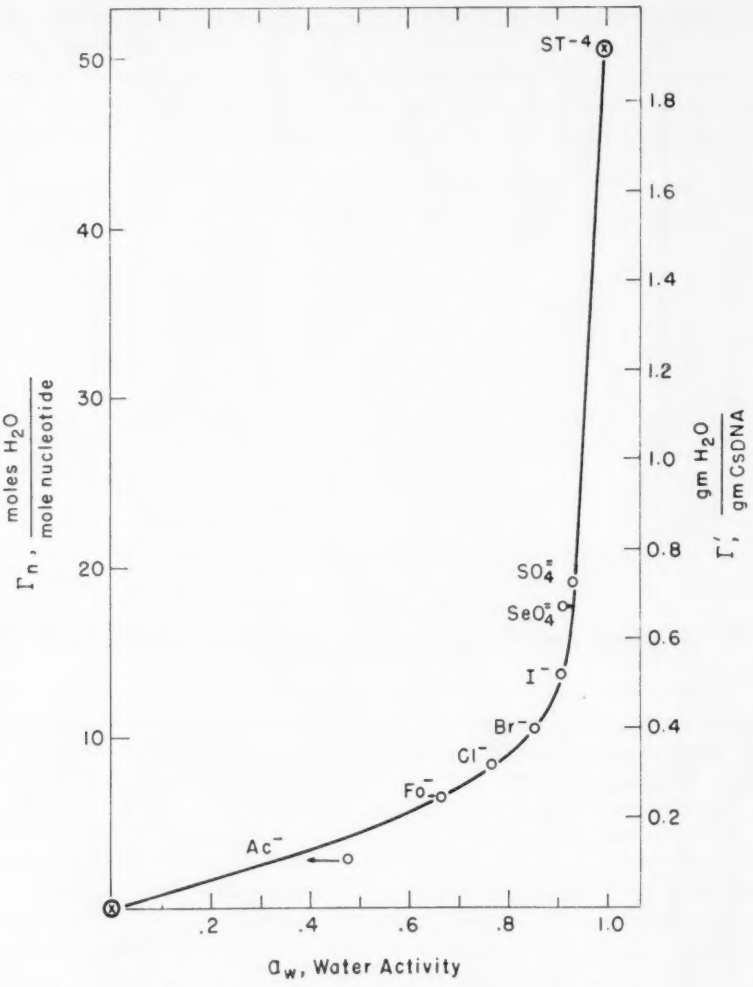

FIG. 4.—The net hydration for T-4 bacteriophage CsDNA at 25°C.

salt solutions are plotted against water activity, again calculated from data in Robinson and Stokes.[9] Osmotic coefficients for points shown with arrows are not available at present. For these, water activities were calculated assuming an osmotic coefficient of one. The direction of the arrow indicates the way the point is expected to move when current isopiestic measurements on these solutions are completed. The data in this paper will be presented with the corrected activity data in a later publication together with the theory of solvation in the density gradient system.

The extrapolated value of CsDNA (Fig. 3) at water activity a_w equal to zero was calculated from the linearly extrapolated LiDNA value (Fig. 2) using an average

nucleotide ion residue weight of 340.[11] The specific volume of the DNA ion was calculated from the extrapolated LiDNA buoyant density assuming the lithium ion added weight but no volume to the DNA. The maximum error resulting from this assumption is 2 per cent of the specific volume. The buoyant density for CsDNA at $a_w = 0$ was then calculated using the difference in the molar volumes of the Cs ion and the Li ion obtained from the difference in the crystal molar volumes of CsBr and LiBr. In these concentrated salt solutions, partial molar volumes and crystal molar volumes are almost identical; consequently little error results from this procedure. The value obtained in this calculation was $2.117 \pm .040$ gm/cc. It should be pointed out that this number is the reciprocal of the specific volume of unhydrated CsDNA. We have learned[13] that the reciprocal of the partial specific volume of CsDNA in water is 2.24 gm/cc. Comparison of the two numbers yields a valuable method of determining the density of the hydrated water at $a_w = 1$ and suggests that the hydrated water has nearly the same density as the water in the solution.

Figure 3 also shows 2 RNA points. The Cs formate point was taken from Davern.[14] The Cs_2SO_4 point was measured by the authors at pH 5.5.[15] These points suggest that there is a small difference in the solvation of RNA and DNA,[16] but the data are too incomplete to make a definite statement. The extrapolated value for anhydrous CsRNA is expected to be less than 0.1 density units greater than the value for CsDNA.

LiDNA has been banded in lithium silicotungstate at pH 4.7. The buoyant density of lithium DNA in this solution was 1.138 gm/cc. The water activity calculated for this solution assuming an osmotic coefficient of one is 0.995. The amount of water per nucleotide was calculated from the extrapolated LiDNA density at $a_w = 0$. The buoyant density was then corrected to CsDNA (Fig. 3) assuming that CsDNA and lithium DNA are equally solvated.

Figure 4 shows Γ_n against a_w. Γ_n is defined as moles water per mole average nucleotide and was calculated[17] for an average nucleotide ion residue weight of 340, assuming the density of the solvated water to be 1.00 gm/cc.

Hydration and dehydration have long been suspected of having an important role in the control of cellular replication.[18] The rapid change in hydration of DNA between $a_w = 0.9$ and $a_w = 1.0$ indicates that such mechanisms should be seriously examined.

Our sincere thanks are extended to F. Bonhoeffer and H. Schachman for permitting us to publish the partial specific volume of CsDNA. We wish also to thank Dr. P. O. P. T'so[19] for the RNA sample.

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‡ Predoctoral Fellow of the National Science Foundation.

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[16] An explanation for at least part of the large differences in buoyant density of CsDNA and CsRNA in CsFormate and CsCl solutions is the fact that the water activity of each of these solutions is such a strong function of solution density.

[17] Γ_n was calculated with the equation

$$\frac{1}{\rho_0} = \frac{v_{\mathrm{DNA}} + \Gamma'_{\mathrm{H_2O}}}{1 + \Gamma'}$$

where Γ is the solvation expressed on a weight basis. The specific volume of the solvated water, $v_{\mathrm{H_2O}}$, is taken to be one, and v_{DNA} is the extrapolated specific volume of CsDNA at $a_w = 0$.

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*TYPES AND FREQUENCIES OF HUMAN CHROMOSOME ABERRATIONS INDUCED BY X-RAYS**

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In spite of widespread concern over the genetic hazards to human populations of ionizing radiations, relatively few quantitative data based on direct determinations of the genetic effects of radiations on human beings are as yet available. The difficulties inherent in genetic studies with individuals or populations exposed to ionizing radiations is evident in the reports of Neel and Schull[1] dealing with the Japanese populations exposed at Hiroshima and Nagasaki. Hence, alternative approaches to this problem seem highly desirable. One such approach is now possible as a result of recent advances in mammalian tissue and cell culture techniques. These advances make possible the initiation, prolonged maintenance with active growth, and effective chromosomal study of normal euploid human cell cultures. Thus, these developments have made available experimental material suitable for studies of radiation-induced aberrations in human chromosomes.

The present paper is concerned with qualitative and quantitative analyses of X-ray-induced aberrations. Previous to the initiation of radiation studies, considerable time was devoted to the development of suitable cytological and culturing techniques. In particular, such studies made possible the construction of a detailed idiogram of the human karyotype,[2] which facilitates the recognition of individual chromosomes. This information has proved to be essential for the accurate analysis of radiation-induced aberrations, as will be discussed in more detail in this paper.

Related investigations of radiation effects on human cells and chromosomes have been performed by Puck and his associates,[3, 4] and by Bender.[5, 6] The former investigators have determined X-ray survival curves of single human cells having either normal or heteroploid karyotypes. Puck[4] has also attempted to correlate his determination (with cultured human euploid cells) of the mean lethal dosage for colony formation with the mean dose required to produce chromosome abnormalities. Lindsten[7] and Fraccaro[8] have published preliminary reports on their collaborative study of radiation-induced post-metaphase aberrations in normal human diploid cells in culture. Brief reports of the present studies have also appeared previously.[9, 10]

Materials and Methods.—In the present experiments, euploid human somatic cell lines in culture were employed. These cultured materials included some epithelioid cells derived by kidney biopsy from a 21-year-old woman but were principally fibroblast-like cells from a number of different sources: skin-muscle tissues of a 2-month old fetus, a 0.5 mm-long aborted total fetus and prepuce biopsies from several newborn children.

General methods of cell culture and cytological examination have been described previously.[2] The only modification of culturing technique has been the use, in recent experiments, of a fetal calf serum-supplemented medium described by Puck and his associates.[11] In certain experiments, cell lines were used which had been maintained as actively proliferating cultures of diploid constitution for 4 to 6 months, although most experiments were performed on cultures within a few weeks after initial biopsy.

For purposes of irradiation, 1 ml of a suspension containing from 1 to 3 $\times$ 10^5 cells was inoculated into depression test tubes (Leighton tubes) holding removable, clean, sterile 30 $\times$ 11 mm cover slips, and incubated at 37°C for 24 hours or more before treatment. During X-irradiation, these tubes were placed with the cover slip side (cell layer side) up in a specially designed constant temperature chamber mounted on an adjustable turntable on casters. A constant temperature (37°C) equivalent to normal human body temperature was maintained before, during, and after irradiation, except for the short time required for handling the tubes and for medium changes. Experimental and control tubes received identical treatment except for the specific radiation exposures. After irradiation, the nutrient medium in all tubes, including the control series, was immediately replaced with fresh pre-warmed solution.

Radiation doses were administered with a General Electric Maxitron operated at 250 kv. Amperage was varied from 3.75 to 30 ma. Filtration consisted of 1 mm of aluminum, and 3 mm of the plexiglass cover forming the top of the constant temperature chamber. A constant exposure time of one minute was maintained for all doses; different doses were administered by varying the amperage and/or distance from target. Dosages were checked with a recently calibrated Victoreen 100 r thimble ionization chamber before each experiment, and at regular intervals during each experiment. In order to measure as accurately as possible the actual doses received by the cells the chamber was placed inside the Pyrex glass Leighton tubes.

Preliminary experiments had helped to determine the desirable time intervals between irradiation and cell fixation, colchicine concentration and length of treatment, method of cytological preparation, etc. In general, with the present dose-range from 0 to 100 r, cells were fixed at intervals of 18, 25, 30, 42, and 67 hours after irradiation. A final concentration of 2.5 $\times$ 10^{-6} *M* colchicine solution was added to all culture tubes from 8 to 15 hours before fixation. Hypotonic

saline pretreatment was employed to spread chromosomes. Slides for aberration analysis were prepared by the aceto-orcein method.

The effect of X rays in inhibiting mitosis was determined by counting the number of cells at metaphase in both the control and treated series at different intervals following irradiation. No systematic attempt has been made to determine the relative sensitivity of various mitotic stages to X rays. The estimates of mitotic activities are based on a random sampling of more than 2000 cells from two separate replicate cultures in each experimental series.

In scoring aberrations, only intact euploid cells were utilized in which all 46 chromosomes (or 46 centromeres with the expected number of accompanying acentric fragments) could be accounted for. The necessity for a thorough acquaintance with the normal complement soon becomes apparent in attempting accurate analyses of cells with many relatively small (1.5 to 10 μ long) chromosomes. In many instances, a decision as to the type of aberration present is possible only following the identification of certain specific chromosomes of the normal complement. In the present studies, detailed free-hand drawings were made of every cell analysed and each analysis was later confirmed by consultations involving two and often three different observers.

Mitotic Inhibition.—The percentage of metaphases in the untreated (control) series was about 1 to 1.5 per cent in active cultures during the first 2 to 5 days of incubation after inoculation. The percentages of mitotic inhibition by different doses of X rays as compared with the untreated controls are shown in Figure 1. The results in this figure are based on the average of 6 experiments for the

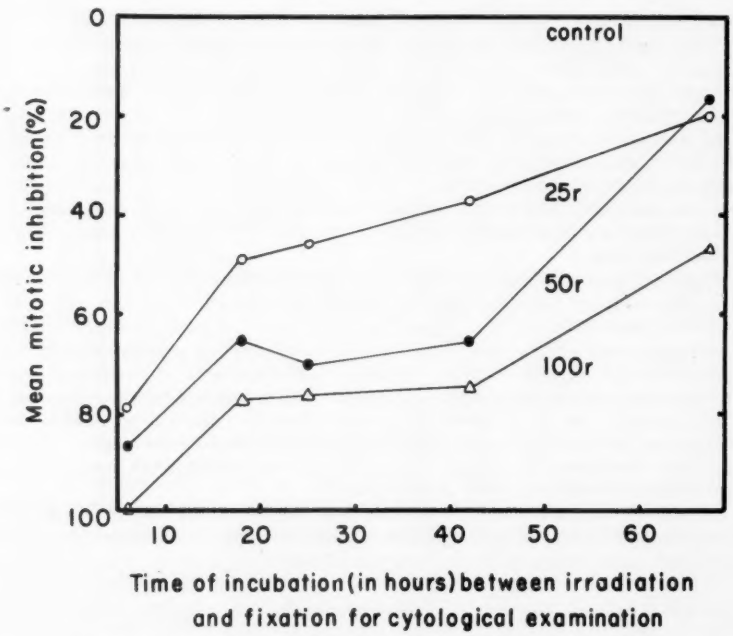

FIG. 1.—Relative mitotic inhibition produced by various X-ray doses.

25-, 42-, and 67-hour series, 3 experiments for the 18-hour series and one experiment only for the 6-hour series. It is evident that the degree of mitotic inhibition is proportional to the dose and that initial inhibition is followed by gradual recovery with time. These data also indicate that at 67 hours after irradiation, recovery

is not complete even at the lowest dose of 25 r. Furthermore, a dose higher than 100 r can inhibit mitotic activity so severely that metaphase configurations become too few for an efficient analysis of aberrations. Although these results are preliminary and more information is necessary before any definitive conclusions may be drawn, the present information on inhibition has proved useful in planning and evaluating the experiments on dosage response curves.

Types of Aberrations and Their Temporal Distribution.—The presently available data from 18 different experiments are presented in Table 1. Cell cultures originating from normal tissues of 5 different individuals were used. There is no indication in the data obtained to date either that cells from different individuals or that different cell types from a single individual differ significantly in radiosensitivity. Hence the data have been pooled.

TABLE 1

SUMMARY OF CHROMOSOME ABERRATION TYPES IN CONTROL AND X-IRRADIATED HUMAN SOMATIC CELLS IN CULTURE

Time after exposure (hours)	Dose (r)	No. of cells scored	Aberration Types: Chromatid: Terminal deletions	Chromatid: Isochromatid breaks	Chromatid: Exchanges	Chromosomal: Terminal deletions	Chromosomal: Interstitial deletions	Chromosomal: Exchanges	Total aberrations	Aberrations per cell
18	0	45	4	0	0	0	0	0	4	0.089
	25	58	19	22	4	0	0	0	45	0.776
	50	35	12	19	0	0	0	0	31	0.885
	100	19	9	22	5	0	0	0	36	1.894
25	0	156	6	2	2	0	0	0	10	0.064
	25	91	18	13	1	0	0	0	32	0.352
	50	131	50	73	7	4	1	2	137	1.045
	100	85	50	135	21	2	3	4	215	2.529
30	0	42	4	1	0	0	0	0	5	0.119
	25	58	6	5	0	3	6	0	20	0.345
	50	34	2	7	0	1	5	1	16	0.471
	100	22	8	22	1	8	17	10	66	3.000
42	0	132	17	1	0	0	0	0	18	0.136
	25	96	17	7	1	4	2	3	34	0.354
	50	62	4	3	0	8	6	4	25	0.403
	100	41	6	1	0	10	17	10	44	1.073
67	0	45	5	0	0	0	0	0	5	0.111
	25	70	8	2	1	8	8	2	29	0.414
	50	42	0	0	0	11	7	4	22	0.500
	100	29	1	0	0	3	11	6	21	0.724
Total scored		1,293								

Qualitatively, these studies indicate clearly that the types of aberrations induced by irradiation of human somatic chromosomes are the same as those found in

TABLE 2

COMPARATIVE FREQUENCIES OF CHROMATID AND CHROMOSOME TYPES IN HUMAN SOMATIC CELLS EXAMINED AT VARIOUS TIMES AFTER X-IRRADIATION

Time after exposure (hours)	Treated cells examined (number)	Induced Aberrations: Chromatid	Induced Aberrations: Chromosome	Induced Aberrations: Total	Control cells examined (number)	Spontaneous aberrations (chromatid) per cell	Per cent induced chromatid aberrations (corrected for spontaneous aberrations)
6	5	9	0	9	...	...	100.0
15	8	14	0	14	...	...	100.0
18	112	112	0	112	45	0.09	100.0
25	307	368	16	384	156	0.06	95.8
30	114	51	51	102	42	0.12	41.4
42	199	39	64	103	132	0.13	16.8
67	141	12	60	72	45	0.11	0
	Total 886				Total 420	Mean 0.10	

comparable experiments with other organisms, such as, for example, the classical studies employing Tradescantia microspores.[12, 13] Both chromatid and chromosome aberrations occur, and essentially all the expected types in both categories have been found. Furthermore, the temporal distribution of these two major categories (Table 2 and Fig. 2) corresponds in general to the pattern anticipated on the basis of previous studies. With low doses (25 and 50 r), chromatid aberrations can be observed within 6 hours after irradiation. Cells examined at 18 hours after irradiation have only chromatid aberrations; at 25 hours a few chromosome types are present, and by 30 hours both categories are present with approximately equal frequencies; by 67 hours, if a correction is made for the incidence of spontaneous aberrations (all chromatid types) in controls, only chromosome aberrations are present in irradiated cells. It is of some interest to note that at the 30-hour period when both categories of aberrations are found in different cells in the population, these two categories were also present, in a few instances, in the same cell.

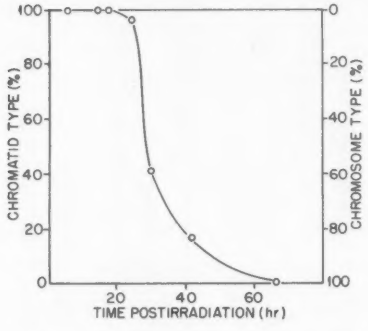

FIG. 2.—Distribution of aberration types (average of all doses) during post-irradiation periods.

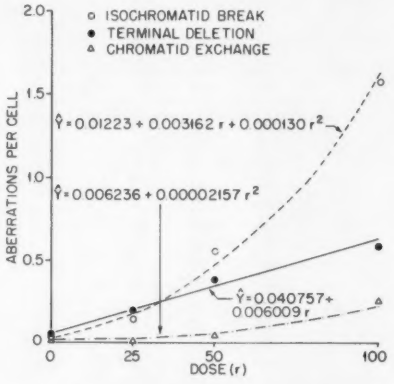
ISOCHROMATID BREAK
TERMINAL DELETION
CHROMATID EXCHANGE
Ŷ = 0.01223 + 0.003162 r + 0.000130 r²
Ŷ = 0.006236 + 0.00002157 r²
Ŷ = 0.040757 + 0.006009 r
ABERRATIONS PER CELL
DOSE (r)

FIG. 3.—Relation between X-ray dosage and frequencies of chromatid aberrations in cells fixed at 25 hours after irradiation. Time of exposure constant (one minute).

For chromatid types, only terminal deletions, isochromatid breaks and the various kinds of inter- and intra-changes were scored. The classification of so-called "achromatic lesions" is ambiguous, since their detection depends, among other things, on the stage of cell division, the distribution of eu- and heterochromatic regions in the chromosomes, and staining reactions. Since many so-called achromatic lesions are not complete breaks, and all degrees of intergradation appear to exist, these lesions were not included in scoring. In the category of chromosome aberrations the following types have been scored: terminal deletions, interstitial deletions, and exchanges such as rings, and dicentrics.

Spontaneous Aberrations.—The frequency of spontaneous chromosome aberrations appears surprisingly high, with an average of 0.1 aberration per cell in all postirradiation periods (Table 2). This frequency is about 10 times higher than that reported by Bender[5] but about one-half of that reported by Puck.[4] Almost all aberrations of spontaneous origin were chromatid deletions and isochromatid types, the only exception being two instances of chromatid exchanges in two cells

out of 420 control cells examined. No aberrations of the chromosome category have been observed in untreated cells. The occurrence of exclusively chromatid aberrations in untreated cells is similar to those reported in Tradescantia,[14] in Chinese hamster embryonic fibroblasts[6, 15] and mouse embryonic cells[16] in culture, and probably in two cell lines of rat sarcoma.[17]

Frequencies of Induced Aberrations.‡—The quantitative data obtained in the present experiments also permit certain conclusions to be drawn concerning dose-effect relationships. The most extensive and reliable data to date are those from the analyses made at 25 hours after irradiation, at which time aberrations are almost all (95.8%) chromatid types. The data for chromatid aberrations at this period are summarized in Table 1 and plotted graphically in Figure 3. Results of one analysis made at 42 hours after irradiation show that 83.2 per cent of aberrations are of chromosomal type. The data for chromosome aberrations at this time period are also given in Table 1 and plotted in Figure 4.

The present quantitative determinations indicate that for both chromatid and chromosome aberrations two major kinds of dose-effect relationships exist. In general, terminal deletions increase linearly with dose, isochromatid breaks consist of both linear and quadratic terms, whereas exchanges exhibit an exponential relationship compatible with a two hit curve.

Discussion.—The present studies indicate that the effects of ionizing radiation in producing aberrations in human chromosomes in tissue-culture cells are fundamentally similar, both qualitatively and quantitatively, to those observed in comparable studies with other cells—both animal and plant. The same two major categories of aberrations, chromatid and chromosome, are observed and these exhibit a similar temporal sequence to that observed previously, i.e., chromatid types constitute the only category observed in the first cells entering metaphase following irradiation with chromosome types appearing at later periods after irradiation. The dose-frequency relationships are, in general, also similar to those observed in other cells such as Tradescantia microspores. Two distinct types of curves have been found: those in which aberration frequencies increase linearly with dose (for both chromatic and chromosome terminal deletions) and those in which aberration frequencies increase approximately as the square of the dose (for both chromatid and chromosome exchanges). The former relationship is expected to hold for aberrations arising as a result of single events (hits), while the latter should hold for those resulting from two separate events (hits) when the time of irradiation is kept constant, as was done in the present experiments. The dose-frequency relationship observed with exchanges strongly implies the existence of an intensity effect in the production by radiation of this type of aberration in human chromosomes, although proof of such an effect would require additional experimental evidence.

The data obtained in these experiments also suggest that the majority of both isochromatid aberrations and chromosome interstitial deletions are the result of two hits. The existence of an appreciable two-hit component in the origin of both these types is well established in Tradescantia.[18, 19]

The present experiments clearly indicate the existence of marked variations in the radiosensitivity of human chromosomes during the mitotic cycle on the basis of observed variations in the frequencies of induced aberrations. This evidence

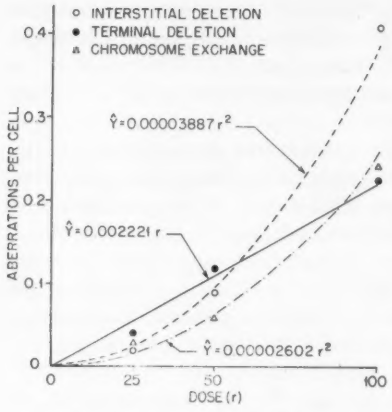

FIG. 4.—Relation between X-ray dosage and frequencies of chromosome aberrations in cells fixed at 42 hours after irradiation. Time of exposure constant (one minute).

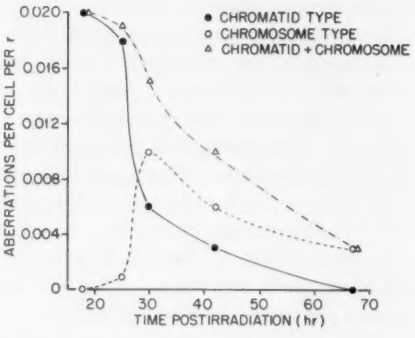

FIG. 5.—Relative radiosensitivity during mitotic cycle as expressed in terms of aberration (chromatid and chromosome) frequencies in cells fixed in various postirradiation periods.

is summarized graphically in Figure 5 (based on data in Table 1), in which aberration frequencies per cell per roentgen (average of all doses) are plotted against postirradiation period (expressed as hours after exposure at which cells have been fixed and scored). The graph indicates the initial presence of only chromatid types which decrease rapidly in frequency and are eventually replaced by only chromosome types.

A pool of the combined frequencies of all aberration types indicates a fivefold decrease in radiosensitivity during the postirradiation period studied. In general, these results agree well with previous studies on variations in the radiosensitivity of cells during the mitotic cycle in demonstrating a much higher frequency of aberrations in cells irradiated after chromosome duplication (which are the first cells to enter metaphase after irradiation and contain only chromatid aberrations) as compared with cells irradiated during the interphase period when the chromosomes are effectively single and only chromosome aberrations are produced.

Observations made in connection with these studies of radiation-induced aberrations also indicate a pronounced effect of X rays in inhibiting mitosis in human cells. The possibility that such inhibition effects may complicate interpretations of variations in radiosensitivity as well as of dose-aberration frequency relationships must be kept in mind. There is some evidence in the present studies that the period of maximum sensitivity to radiation (as judged by total aberration frequencies) is dose dependent, suggesting the possibility that cells exposed to higher doses (and having higher aberration frequencies) may be relatively more delayed in entering metaphase. Such an effect could distort comparisons based on cells exposed to different doses but fixed and scored at a constant time period after irradiation. However, if this effect is pronounced, it should be reflected in a dose-dependent transition from chromatid to chromosome aberration types, such that the appearance of chromosome types should be markedly delayed in cells exposed to higher doses. Since no such effect is observed in the present studies, it appears

appropriate to compare the effects of different doses at the same time period after irradiation.

The quantitative data obtained in these experiments can be used to estimate the sensitivity of human chromosomes to ionizing radiation—a major goal of these studies. This sensitivity may be expressed in various ways, e.g., in terms of aberrations per cell (or per chromosome) per roentgen, or of breaks per cell (or per chromosome) per roentgen. It has been shown (Fig. 5) that the most radiosensitive period of the cell comes after the chromosomes are effectively split. Under the experimental conditions, these cells were fixed at 18 hours. At this period the aberration frequency per cell per roentgen was calculated to be 0.02, about 5 times higher than in cells at interphase which were fixed at 67 hours after irradiation. For the sake of comparison, these figures can be expressed in terms of breaks per cell per roentgen. Thus, the figures have been calculated by averaging aberration frequencies induced by different doses (Table 1) to be 0.022 at 18 hours and 0.015 at 67 hours (if isochromatid aberrations are considered to result from one break), or 0.034 and 0.016 respectively (if isochromatid aberrations are considered to result from two breaks). Similar independent studies by Bender[5, 6, 22] and Puck[4] have shown that the aberration frequencies in human euploid somatic cells were 0.003 breaks/cell/r and 0.027 hits/mitosis/r, respectively. Lindsten[7] reported for human embryonic lung and brain cells in culture an average number of chromosome hits per mitosis per rad as 0.008–0.14. The present data appear to be in the same order of magnitude as that estimated by Puck. Nevertheless, it should be noted that the latter results were derived from a pooled sample of cells which were fixed from a few minutes to seven days after irradiation. Furthermore, in the same data aberration categories such as sticky chromosomes and achromatic lesions (which are dose-independent) have been included.

Bender[6, 20] maintained that fibroblast-like cells differ in radiosensitivity from the epithelioid cells. Lindsten[7] and Fraccaro,[8] on the other hand, observed that the "spontaneous" aberration frequency was lower in lung (0.6–0.9%) than in brain cultures (1.5–3.0%). Their primary brain cultures constantly displayed two types of cells whereas the primary lung cultures displayed mostly fibroblast-like morphology. In the present study, about 10 per cent of cells were epithelioid, derived from kidney biopsy. No significant difference in radiosensitivity was observed among cell types. Additional comparative studies of different cell types would appear necessary to clarify the situation.

Finally, the question whether or not human chromosomes are more radiosensitive than those in other organisms may be considered. When the coefficient of

TABLE 3

COEFFICIENTS OF X-RAY-INDUCED ABERRATIONS IN HUMAN SOMATIC CELLS *in vitro* AND IN TRADESCANTIA MICROSPORES[22]

Cells	Chromatid breaks per cell per r $10^{-2} \times$	Isochromatid breaks per cell per r $10^{-2} \times$	Chromatid exchanges per cell per r^2 $10^{-3} \times$
Tradescantia microspores	0.725 ± 0.08	0.271 ± 0.02	1.81 ± 0.21
Human somatic cells *in vitro*	0.601 ± 0.067	0.316 ± 0.186	2.157 ± 0.496

chromosome aberration production by X rays in human cells *in vitro* is compared with that in Tradescantia (Table 3), it is evident that these mammalian and plant cells show similar radiosensitivities, in support of the view that the mechanics of

chromosome aberration production are essentially the same in both plant and mammalian cells. Bender and Wolff[22] have shown, based on the data[6] from samples in the first postirradiation division, that mammalian chromosomes actually exhibit an even lower sensitivity than plant chromosomes. However, as pointed out by these authors, the seemingly low values for the coefficients of aberration production in their results and in our results should in no wise be interpreted as being a measure of the amount of genetic damage inflicted on cells. The relation of observable chromosome aberrations at metaphase to primary breaks, detrimental point mutations, and cell killing is complex and still obscure.

Summary.—Qualitative and quantitative analyses have been made of X-ray-induced chromosome aberrations in human euploid cells grown in culture. Two major categories of aberrations—chromatid and chromosome—are present in irradiated cells. Chromatid types constitute the only aberrations in cells observed shortly after irradiation; chromosome types begin to appear and finally completely replace the former in successively later post-irradiation periods. A general decrease in radiosensitivity occurs in conjunction with this temporal shift in the appearance of aberration types. Quantitative results for both the chromatid and chromosome aberration types indicate that, in general, terminal deletions increase linearly with dose, whereas exchanges exhibit an exponential relationship.

The frequency of spontaneous aberrations, which are almost exclusively one-hit chromatid types, is on the average 0.1 aberrations per cell in all postirradiation periods. The induced frequency at the most radiosensitive period of the cell (which follows the period during which the chromosomes become effectively double) is 0.02 aberrations per cell per roentgen. Such cells are about 5 times more sensitive than cells at interphase when chromosomes are effectively single.

The coefficient of chromosome aberration production by X rays in human somatic cells is shown to be not significantly different from that in certain plant cells. This conclusion supports the view that the mechanics of chromosome aberration production by radiation are similar in both plant and mammalian cells. However, this result cannot be taken to mean that radiosensitivity—in terms of genetic damage, mutation production, and cell killing—is necessarily the same in plant and mammalian, especially human, cells.

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*INDUCTION OF ALKALINE PHOSPHATASE BY SUBSTRATES IN ESTABLISHED CULTURES OF CELLS FROM INDIVIDUAL HUMAN DONORS**

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Many microbial systems of inducible and repressible enzyme synthesis are known.[1–4] A few examples of inductive and repressive effects have been described in cells of higher organisms in tissue culture.[5–9] In most of these cases, the difference between maximum activity, induced or repressed, and control activity is not very great. The present communication describes the induction in established cultures of human skin fibroblasts of alkaline phosphatase activity (presumably *via* enzyme synthesis) by putative substrates of the enzyme. An advantage of this alkaline phosphatase system is that cells grown in the absence of inducer have little or no detectable enzyme activity, while induced cultures show very substantial activity, 30 to 50 fold greater than the minimum detectable by our methods.

That alkaline phosphatase synthesis in homeotherm cells might be based on an inducer-repressor system is suggested by embryological evidence.[10, 11] During early embryonic development in the chicken, all cells contain alkaline phosphatase.[10] Enzyme activity decreases in most tissues to undetectable levels during the intermediate stages of development. Later, it reappears in the organs and tissues which in the adult normally contain the enzyme.[10,11] These include the intestinal epithelium, proximal tubule of the kidney, osteoblasts, granulocytes, liver cells, and a few others. However, the enzyme is "absent" in most other tissues such as the fibroblasts of the skin, though it may appear under special circumstances, such as healing skin wounds, and in certain tumors derived from tissues which do not otherwise show it.[12–14]

The presence of alkaline phosphatase activity in all cells of the early embryo

and its restricted or conditional location in the adult suggests that it is based on an inducible system. This is known to be so in microorganisms,[2] in explants of amphibian,[15] avian, and mammalian embryonic intestine,[11] and in human granulocytes *in vivo*.[16] In microorganisms, alkaline phosphatase synthesis is induced when the concentration of one product of its activity, inorganic phosphate, is reduced in the medium.[2] In explants of embryonic amphibian, avian, and mammalian intestine, alkaline phosphatase synthesis is induced at an earlier stage than normal by either adrenal glucocorticoids or certain presumed substrates of the enzyme.[11, 15] Human granulocyte alkaline phosphatase is markedly increased in patients treated with hydrocortisone.[16] Cox and MacLeod[9] have shown that a hydrocortisone analogue, prednisolone, induces alkaline phosphatase activity in some but not all established human "epithelial" cell lines. The present communication describes the substrate induction of alkaline phosphatase activity in established human skin "fibroblastic" cultures from individual healthy donors.

Methods.—Cell cultures used for the present work are from strains available in this laboratory and originally established either by trypsinizing with a modified Younger technique[17] or by explanting small pieces of tissue. All but one strain are derived from foreskins of children. The exception (HR) is from the forearm of a healthy female donor. They are uncloned, grown on glass surfaces in Waymouth's medium with 10 per cent heated calf serum, and kept in culture by successive trypsinizations. For each experiment, the cells of a strain were pooled from several bottles, and the same cell numbers from this pool were inoculated into each of the bottles required for the experiment. The only known difference between bottles of one experiment was the addition of putative substrate or special medium. The substrates used were disodium phenylorthophosphate (Hopkin & Williams Ltd.) or disodium β glycerophosphate (Eastman Kodak Ltd.).

Cells were harvested by trypsinization and counted in triplicate in a Levy hemocytometer. The cells were twice washed in saline and lysed in 1% aqueous sodium deoxycholate at a concentration of 5 million cells/ml. Alkaline and acid phosphatase were determined by a modification of Huggins and Talalay phenolphthalein phosphate method.[18] Magnesium chloride at a final concentration of 0.003 *M* was added to the substrate. To reduce the number of cells required, a semimicro method using 0.1 cc of deoxycholate lysate and 0.4 cc of substrate was used. Incubation was at 37°C and pH 9.75 for 4 hr in order to increase the accuracy in cases where low levels of alkaline phosphatase were present. Colorimetric determinations were made immediately after adding 0.4 cc glycine buffer to the reactants, employing a Unicam SP.600 spectrophotometer with semimicro cuvette at 540 mμ. alkaline phosphatase results are expressed as mg of phenolphthalein liberated by 5 million cells during 4 hr incubation at 37°C. The amount of phenolphthalein released was proportional to alkaline phosphatase at concentrations of 0.2 mg per ml phenolphthalein or greater in a 4-hr period. Below this concentration of phenolphthalein, enzyme activity was not proportional to enzyme concentration. This sets the lower limit of accurate measurement of enzyme activity in our experiments.

Experimental Results.—Nine individual human skin cell strains were studied from 6 to 15 months after the cultures were established. All nine cell strains lacked alkaline phosphatase activity when cultured in the usual manner (Table 1) (i.e., with inocula of 200,000 or more cells in a 6-oz medicine bottle with 10-ml medium, so that subculturing is required every 7 to 12 days). As previously reported,[9] prednisolone (phosphate or hemisuccinate) at concentrations of 0.5 μg/ml did not influence the alkaline phosphatase activity of the skin fibroblast strains studied (Table 1). On the other hand, the results of the present experiments show that high alkaline phosphatase activity is induced by both of two methods in seven out of nine fibroblast strains (Table 1). One of the two remaining strains "Wil" is induced by one method but not by the other, and the other strain "Fio" is not induced by either.

TABLE 1

ALKALINE PHOSPHATASE ACTIVITY AMONG CULTURES OF ESTABLISHED SKIN FIBROBLASTS FROM INDIVIDUAL DONORS

Donor	Duration of cultivation, months	Chromosome number	Alkaline Phosphatase Activity: Without induction	With prednisolone	With phenyl-phosphate	In own old medium
Cla	8	46	−	−	+	+
Gey	8	46	−	−	+	+
Kel	8	...	−	−	+	+
Mas	8	46	−	−	+	+
Kut	8	...	−	−	+	+
H.R.	6	...	−	...	+	+
Hal	8	...	−	...	+	+
Wil	15	...	−	...	+	−*
Fio	12	...	−	−	−	−

+ Alkaline phosphatase activity present.
− Alkaline phosphatase activity absent.
... Not tested.
* Induced by old conditioned medium from an inducible strain (Mas).

TABLE 2

INDUCTION OF ALKALINE PHOSPHATASE ACTIVITY IN HUMAN FIBROBLASTS GROWN IN THE PRESENCE OF PHENYLPHOSPHATE FOR 7 TO 15 DAYS

Cell strain	Number of experiments	Alkaline Phosphatase Activity*: Cells ($5 \cdot 10^6$) grown without phenylphosphate	Cells ($5 \cdot 10^6$) grown with phenylphosphate
Mas†	7	<0.02	0.40
Cla†	4	<0.02	0.40
Kel†	1	<0.02	0.33
Gey†	2	<0.02	0.14
Kut†	1	<0.02	0.40
H.R.†	1	<0.02	0.25
Hal	1	<0.02	0.27
Wil	1	<0.02	0.20
Fio†	2	<0.02	<0.02

* Milligram of phenolphthalein released by a deoxycholate lysate containing $5 \cdot 10^6$ cells. Incubation at 37° for 4 hr.
† Other experiments using qualitative methods gave similar results.

The first method is the growth of cell cultures in the presence of organic monophosphates (Table 2). Disodium phenylmonophosphate in concentrations in the growth medium ranging from 0.5 mM to 5 mM induces alkaline phosphatase activity in eight of the nine fibroblast strains. Higher concentrations of phenylphosphate inhibit cell multiplication. As seen in Tables 1 and 2, one of the nine strains (Fio) was not induced by growth with phenylphosphate. Figure 1 shows the result of an experiment with the human skin fibroblast strain "Mas." Alkaline phosphatase activity of cultures grown in the presence of 5 mM phenylphosphate is 40 fold greater than the minimum amount detectable by our method. Replicate cultures grown in the absence of inducer for the same time have alkaline phosphatase activity below the sensitivity of the method (0.02

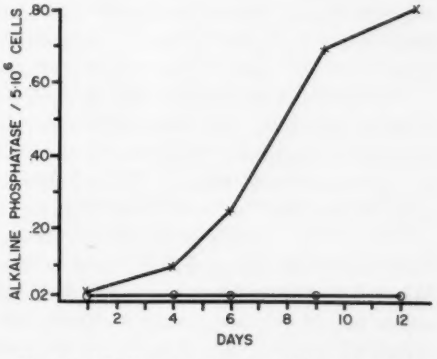

Fig. 1.—Alkaline phosphatase activity of replicate cultures grown with (×) and without (○) 5 mM phenylphosphate (strain Mas).

TABLE 3

COMPARISON OF ALKALINE PHOSPHATASE ACTIVITY IN HUMAN SKIN FIBROBLASTS GROWN FOR NINE DAYS IN THE PRESENCE OF EITHER PHENYLPHOSPHATE OR β GLYCEROPHOSPHATE

Cell strain	Alkaline Phosphatase Activity*		
	No inducer	β glycerophosphate (5 mM)	Phenylphosphate (5 mM)
Mas	<0.02	0.09	0.590
Cla	<0.02	0.05	0.200
Kel	<0.02	0.04	0.130

* See footnote to Table 2.

mg of phenolphthalein). β glycerophosphate disodium salt in equimolar concentrations was less effective than phenylphosphate as an inducer of alkaline phosphatase synthesis (Table 3). Acid phosphatase activity is present in all the cell strains tested. It is not altered by growth in medium with added phenylphosphate.

Unlike bacterial alkaline phosphatase,[2] that of human skin fibroblast cultures is insensitive to the inorganic phosphate concentration of the medium. This concentration is about 1.5 mM in the medium used; reduction to 0.05 mM does not induce activity, and increase up to 5.0 mM does not repress induction by the substrate.

Experiments on HeLa cultures indicate that in only one (Henle) of three HeLa lines (Henle, NIH, and MBA) could phenylphosphate induce alkaline phosphatase activity. Even in the Henle line, induction was possible only with very high concentrations of phenylphosphate (20 mM or greater) and the induced activity was only three to five fold over that of the controls. In all three of the HeLa lines studied, alkaline phosphatase activity was readily induced by prednisolone (0.5 μg/ml).[9]

The second method of alkaline phosphatase induction in human skin fibroblasts is to start a culture with a small inoculum and let the cells multiply to the maximum without changing medium. If 30–60 thousand cells are inoculated in a 6-oz medicine bottle with 15 ml of Waymouth medium containing 10 per cent calf serum, it is possible to keep the culture for at least 20 days without adding or changing medium. Seven of nine skin fibroblast strains developed alkaline phosphatase activity comparable to that following substrate induction when handled in this way (Table 4). Two of the strains (Fio and Wil) did not respond to this treatment. Induction by this not precisely controllable method was observed in about three-quarters of the experiments with the seven "self-inducible" fibroblast strains.

When cells of an inducible strain are inoculated in medium in which cells of the same or another "self-inducible" strain have grown for at least 20 days ("old conditioned medium"), alkaline phosphatase activity appears within 6 to 13 days in eight of nine cell strains. Table 5 shows the results of experiments on four fibroblast strains, two "self-inducible" (Mas and Kel) and two not inducible by "aging" (Wil and Fio). Using qualitative methods, the other five human fibroblast strains (Cla, Gey, Kut, Hal, and H.R.) were readily induced by "old conditioned medium." When cells from an inducible strain are seeded into 20- to 40-day-old medium from either one of the two negative cell strains (Fio or Wil), no alkaline phosphatase synthesis occurs. An observation of particular interest is strain "Wil" which does not develop activity by the "aging" procedure described and does not "condition" for other strains the medium in which it is grown but responds, nevertheless,

TABLE 4

INDUCTION OF ALKALINE PHOSPHATASE ACTIVITY IN HUMAN FIBROBLASTS GROWN WITHOUT CHANGE IN WAYMOUTH MEDIUM FOR 20 TO 40 DAYS

Cell strain	Age of culture, days	Alkaline phosphatase activity*
Mas†	30	0.50
Cla†	23	0.32
Kel†	30	0.15
Gey†	30	0.50
Kut†	24	0.30
H.R.	27	0.40
Hal	33	0.20
Wil†	22	<0.02
	30	<0.02
	40	<0.02
Fio†	19	<0.02
	22	<0.02
	34	<0.02
	40	<0.02

* See footnote to Table 2.
† Other experiments using qualitative methods gave similar results.

TABLE 5

INDUCTION OF ALKALINE PHOSPHATASE ACTIVITY IN HUMAN FIBROBLASTS GROWN IN "OLD MEDIUM" CONDITIONED BY INDUCIBLE CELL STRAINS

Cell strain	Duration of growth, days	Alkaline Phosphatase Activity*: Cells ($5 \cdot 10^6$) grown in fresh Waymouth	Alkaline Phosphatase Activity*: Cells ($5 \cdot 10^6$) grown in "old conditioned" Waymouth
Mas†	7	<0.02	0.375
	8	<0.02	0.250
	10	<0.02	0.400
Kel†	6	<0.02	0.500
Wil	13	<0.02	0.400
Fio†	11	<0.02	<0.02
	13	<0.02	<0.02

* See footnote to Table 2.
† Other experiments using qualitative methods gave similar results.

to "old conditioned medium" obtained from a "self-inducible" strain (Table 5).

Dilution of "old conditioned medium" with an equal volume of fresh Waymouth medium destroys its ability to induce alkaline phosphatase in the inducible strains. An aqueous extract prepared by freezing and thawing a suspension of induced cells when added to cultures did not induce.

Discussion.—The work reported demonstrates induction of alkaline phosphatase in established cultures of human skin fibroblasts by a substrate of the enzyme. The change in enzyme activity is high in comparison to that reported for other inducible or repressible mammalian systems.[5–9] The substances (phenylphosphate and β glycerophosphate) which we have found to be effective for the induction of alkaline phosphatase are substrates *in vitro* for this enzyme as well as for acid phosphatase. They have, however, no effect on the acid phosphatase activity of skin fibroblasts. In animal tissues, alkaline phosphatase is probably a heterogeneous group of enzymes and its *in vivo* enzymatic function and substrate(s) are unknown. In cultured human cells, the apparent inability of inorganic phosphate to repress alkaline phosphatase induction even at high concentrations suggests that the enzymes may not act as hydrolases *in vivo*.

There is a striking difference in the induction of alkaline phosphatase between established cultures of human "epithelial" and "fibroblastic" cells. Most of the former, as shown by Cox and MacLeod,[9] are inducible by a hormone analogue

(prednisolone) but not by low concentrations of substrate. The latter are not inducible by the hormone but respond very effectively to the substrate. Out of nine of the skin fibroblast strains studied, eight were inducible by phenylphosphate. The exception was strain "Fio." Whether this difference reflects variation between donors, variation between the types of cells established in culture, or variation which occurred during culture has not yet been determined. The inability to induce alkaline phosphatase in mammalian cells by means of substrates reported by Klein[7] may have been due either to the cell lines studied or to the concentration of substrate used. As we have shown, some HeLa lines are inducible only by very high concentrations of substrates, others are not inducible, and most skin fibroblasts are inducible by much lower concentrations. The inducibility of skin fibroblast cultures by substrate and the development of activity during wound healing *in vivo*[12] may be related.

When seven of nine skin fibroblast strains are grown from a small inoculum in a relatively large amount of medium for 20 or more days, high alkaline phosphatase activity usually appears. The medium ("old conditioned medium") in which this "self-induction" occurred is able to induce alkaline phosphatase activity promptly in cells of the strain grown in it as well as of other "self-inducible" strains. "Old conditioned medium" can also induce activity promptly in strain "Wil" which is not able to "self-induce" and the old medium of which is not able to induce other strains. Finally, strain "Fio" is not inducible by aging, growth in "old conditioned medium," or substrate. These findings suggest that strain "Wil" is unable to "condition" medium but is able to develop alkaline phosphatase activity, while strain "Fio" is unable to do either. The conditioning of medium may involve either production of one or more inducing substances or removal of one or more inhibitors present in fresh medium: it is perhaps significant that a small dilution of "old conditioned medium" with a fresh one leads to loss of activity.

The conditioning of medium, the determination of restricted distribution of constitutive alkaline phosphatase among different tissues, and the development of the enzyme under special conditions (e.g., wound healing and tumors) are likely to be interrelated processes with an important bearing on differentiation, normal and abnormal.

Summary.—Induction of alkaline phosphatase activity in established (6 to 15 months) human skin "fibroblastic" strains from different donors can be obtained by two methods. One method is by adding substrates of the enzyme to cultures. The other is less consistent and less well defined. It consists of growing a small cell inoculum in a relatively large amount of medium for a long period (20 or more days). The "old" medium is "conditioned" by the induced cells and will induce other susceptible strains. There are variations between human fibroblast strains in their response to either of these methods.

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*THE CHEMICAL AND MUTAGENIC SPECIFICITY OF HYDROXYLAMINE**

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The hereditary changes that convert one base pair (or base) of nucleic acid into another one have been divided into two classes—transitions and transversions.[1] This paper is concerned with the further subdivision of mutagenic transitions. For this purpose, the specific mutagen hydroxylamine[2] (HA) has been employed, which, for the normal DNA bases, reacts with cytosine (C) and 5-hydroxymethylcytosine (HMC) but not or only little with 5-methylcytosine (MeC) or thymine (T). The reaction with C has been examined in detail, since it is apparently responsible for the mutagenic effect of HA. Other bases, e.g., 5-bromouracil (BU), also react strongly with HA, but for BU-containing phages this effect is mainly lethal. In contrast to HA, the chemically similar agent hydrazine reacts much more with T than with C and is much less mutagenic.

The induction of reverse mutations by HA shows a clear bipartition into highly and little inducible phage T4 *r*II mutants; this indicates which base pair is present at the mutant site.

Abbreviations used: A = adenine, HMC = 5-hydroxymethylcytosine, C = cytosine, G = guanine, T = thymine. AP = 2-aminopurine, BU = 5-bromouracil, EES = ethylethane sulfonate, HA = hydroxylamine. CR = cytosine riboside, CdR = cytosine deoxyriboside, etc. dHMP = 5-hydroxymethylcytosine-deoxyribotide. G-C = guanine-cytosine pair in DNA, where C stands for any one of the cytosines.

Material and Methods.—The phage T4 *r*II mutants, bacteriological media, and genetic methods have been described previously.[2-5]

Chemicals: dHMP and glucose-dHMP (of phage T2) were gifts of Dr. Lehman, Stanford University. 2,4-diaminopyrimidine was a gift of Dr. Bendich, Sloan Kettering Institute. All other agents are commercially available.

Mutation induction by hydroxylamine (HA): Method used throughout this paper.

(1) *Reaction mixture:* Reagents: A. 2.5 *M* $NH_2OH \cdot HCl$ plus 2.0 *M* NaCl, kept in cold (up to one week); B. 1 *M* $MgSO_4$; C. 1.5 *M* Na_2HPO_4; D. 10 *N* NaOH. Shortly before use, take 10 ml A, 0.02 ml B, 1 ml C; add D (*ca.* 2 ml) until pH is nearly 7.5; fill to 20 ml volume with distilled water; add more D, cautiously, until pH is 7.5.

(2) *Dilution broth:* Bactotryptone, 5 gm; NaCl, 60 gm; H_2O, 1 liter. The high salt concentration is useful, since without salt even low concentrations of HA inactivate phages very rapidly.[3]

(3) *Stopping mixture:* Dilution broth plus 2 ml acetone per 100 ml medium (gives about 0.4 *M* acetone). This concentration of acetone has no inactivating effect on phages, but it reacts very fast with HA and thereby annihilates it. Nevertheless, it is advisable to plate the phages as soon as possible, since an additional inactivation slowly takes place.

(4) *Reaction procedure:* Take reaction mixture, ice-cold, 2 ml. Add phages, ice-cold, 0.5 ml. Mix for 30 seconds. Take 0.2 ml aliquots into 10 ml ice-cold stopping mixture (= "zero control"). Place remainder in 37°C (= zero time). Take at various times 0.2 ml aliquots into 10 ml ice-cold stopping mixture.

Measurement of the decrease of UV absorption: Reaction mixtures: (*a*) 0.5 *M hydroxylamine* × HCl; 0.04 *M* Na_2HPO_4; 5×10^{-4} *M* $MgSO_4$; pH 7.5. (*b*) 0.5 *M hydrazine* × 2 HCl; 10^{-3} *M* $MgSO_4$; pH 8.5. In most cases, *ca.* 15 μl of a 20 μM/ml solution of the nucleosides or nucleotides were added to 2 ml of reagent mixture such that an OD_{276} of just below 1 was obtained. The mixture was kept at 37°C in glass-stoppered cuvettes (silica greased) and the OD_{276} read against both H_2O and the reagent mixture alone, which was incubated in the same way. We used 0.5 *M* HA and hydrazine, since the 1 *M* concentrations already have an appreciable UV absorption, which significantly decreases during the time of measurement. This self-reaction is accompanied by the production of gas bubbles; more of these bubbles are observed in the presence of one of the bases, indicating their catalytic effect, and therefore, exact measurements of the alteration of the bases themselves become more difficult.

Preparation of BU-containing phages: Both methods of Litman and Pardee[5] and of Freese[4] were used.

Column chromatography: In order to keep the UV absorption of the eluent low, we eluted with dilute H_2SO_4 or 0.05 *M* $NaHPO_4$, at various pH's, off Dowex 50 and with dilute ammonia or 0.05 *M* NH_4 chloride off Dowex 1. Length of column was 10 cm.

Reduction by titanium chloride, $TiCl_3$: The eluent was adjusted to pH 5 and excess phosphate (0.2 *M*). 1/100 volume of a 20% $TiCl_3$ solution was added at room temperature and the precipitate removed. The filtrate was applied to a Dowex 50 column at pH 2.

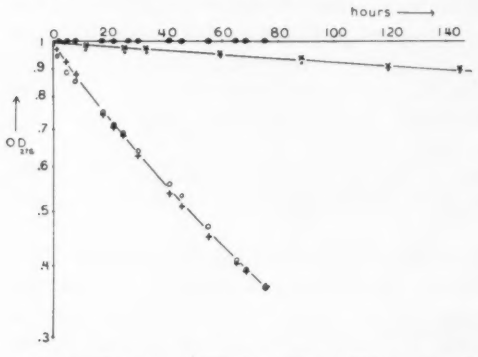

FIG. 1.—Decrease of UV absorption at 276 mμ of various nucleotides in 0.5 *M* HA at pH 7.5. • = dTMP, · = dHMP, × = dHMP − glucose, + = dCMP, O = UMP.

Results.—The reaction of hydroxylamine with the pyrimidine bases: HA reacts with certain pyrimidines[2] and causes the decrease of UV absorption in the range of 260–280 mμ; this is shown in Figure 1 for UMP, dCMP, dHMP, and dHMP-glucose. No reaction has been observed for dTMP, MeC, pseudouridine, or the purines. The thymine analogs BU or BUdR, however, react even faster than the corresponding uracil com-

pounds. Electron-donating (e.g., methyl) groups seem to reduce and electron-attracting groups (e.g., Br) seem to increase the reactivity with HA. This reactivity also increases with the pH.

The HA reaction with U and BU or their ribosides leads to the apparently irreversible loss of the UV absorption peak in the 260–280 mμ range. BU is readily debrominated, as can be observed by the heavy precipitate of AgBr formed when nitric acid and silver nitrate are added to the reaction mixture.

In contrast, the decrease of the UV absorption after HA reaction with C can be almost completely reversed by exposure of the reaction mixture to low pH. Figure 2 illustrates how the UV absorption returns to nearly the same absorbency that was originally observed at low pH by a first-order kinetics and with a rate depending on the pH. Such a reversal can also be observed for CdR but the absorbency does not return completely, even after long times of treatment at pH's 0–1.

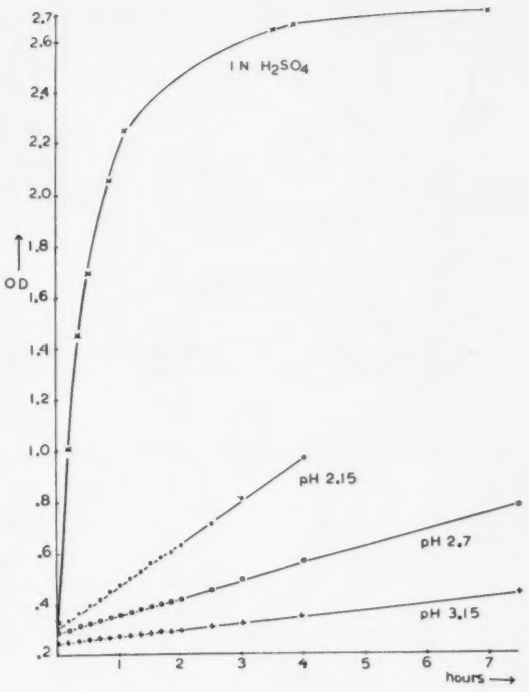

FIG. 2.—Increase of UV absorption at 276 mμ of the pH 7.5 reaction product of C + HA when acidified by H_2SO_4.

When the reaction mixture of C (compound I) with HA is carefully acidified to pH 3 and immediately applied to a Dowex 50 column, the main reaction product (II) elutes at once and can be isolated when care is taken that the pH never drops much lower and that the eluted material is immediately neutralized. Figure 3*a* shows the spectrum of this compound II. Upon acidification, this material recovers a UV absorption peak at about 276 mμ with a first-order kinetics similar to that shown in Figure 2. When compound II is reacted for several hours with 1 *N* HCl and then eluted on a Dowex 50 column (with 0.05 *M* NaH_2PO_4 at pH 3.5), a new compound III comes off. Although its spectrum, shown in Figure 3*b*, closely resembles that of C at acid pH, it differs from it at neutral and basic pH. Also its pK value must be lower (about 3) than that of C (pK 4.6), which readily elutes only when the pH is above 5. Mild reduction of compound III by $TiCl_3$ at pH 5 and room temperature gives rise to a major product (IV) that elutes off a Dowex 50 column at the same time as C and indistinguishable from it when the two are chromatographed together. Also the spectrum of this compound IV (Fig. 3*c*) is the same as that of C.

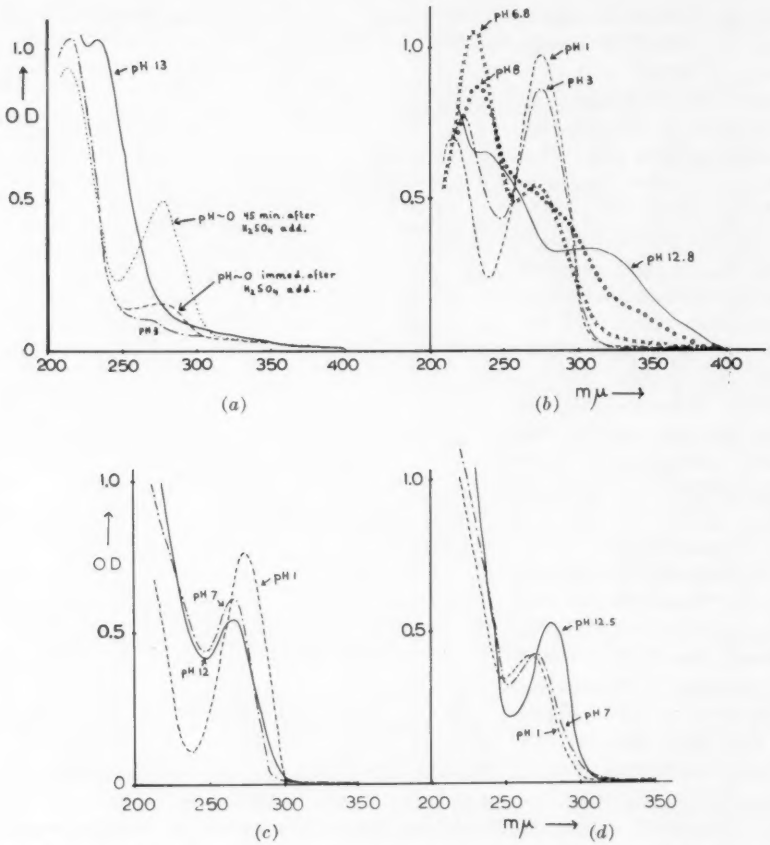

FIG. 3.—UV spectra of various reaction products of C after purification on Dowex 50 cation exchanger.
(*a*) Main product of reaction of C with 1 *M* HA at pH 7.5. = Compound II in Figure 4.
(*b*) Compound (III) obtained (from II) by acidification (pH 0).
(*c*) Compound (IV = I) obtained from the acidified product (III) by reaction with $TiCl_3$ at pH 3. Spectrum is identical with that of C.
(*d*) Spectrum of 2,4-diaminopyrimidine.

One might have expected that HA would predominantly attack the keto group of C and that compound III would be the ketoxime. Our results, however, exclude this possibility as the major reaction, since the reduction of this 2-oximo-4-amino-pyrimidine should result in 2,4-diamino-pyrimidine, which we do not obtain. 2,4-diamino-pyrimidine has absorption spectra shown in Figure 3*d* and elutes from a Dowex 50 column only at very high pH; it is clearly different from compound IV. Hence, we conclude that compound IV is actually C and that the major reaction products of the chemical reactions analyzed above are those given in Figure 4. At present we cannot exclude the alternative possibility that the initial attack of HA breaks the pyrimidine ring between the 3-N and 4-C position, followed by ring closure upon acidification to yield compound III. The initial attack of HA is prob-

ably the same as that of hydrazin, at the 4-carbon of cytosine; for hydrazine, however, the other NH_2 group reacts with the 6-carbon and thus 3-aminopyrazole[7] is produced.

Compound III is quite stable in 2 *N* H_2SO_4 at 100°C, but treatment with 0.5 *N* NaOH for $^1/_2$ hour at 80°C diminishes the UV absorption (partial destruction) and alters it towards that of U. The reaction mixture was applied to a Dowex-1-formate column, and a compound was eluted when the same pH (9.0) was reached at which normally U comes off. Its spectra, at pH's 0, 9, and 14, were also those of uracil. We therefore conclude that high pH easily hydrolyzes the =NOH group, at the 4-position of compound III, resulting in a Keto group, i.e. giving uracil.

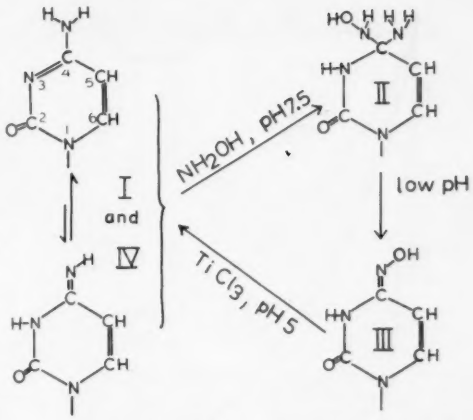

FIG. 4.—Proposed mechanisms of reactions with C, described in the text.

Similar to C, CdR also produces with HA a compound (II) which is immediately eluted off Dowex 50 at pH 3 and upon acidification is converted into another compound (III) with a spectrum similar to that obtained from C (see Fig. 5). Reaction of III at high pH apparently produces UdR. Prolonged reaction of CdR with HA gives rise to some additional compound which elutes only at very high pH and has again a different spectrum.

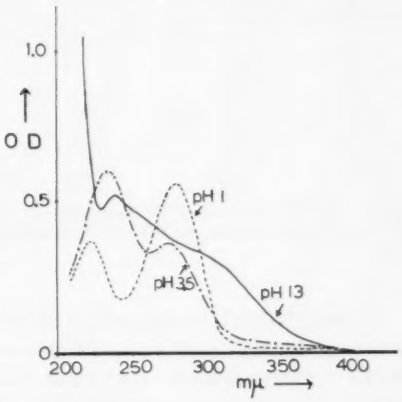

FIG. 5.—UV spectrum of the acidified reaction product (III) of CdR with HA.

The reaction of hydrazin with the pyrimidine bases: Concentrated hydrazin readily reacts with the pyrimidine nucleotides CMP and UMP to give pyrazole derivatives, urea, and ribose phosphate.[7] The reaction with RNA[8] and DNA[9] produces "apyrimidinic acid." We have examined the decrease of the UV absorption of various nucleotides caused by the reaction with hydrazin under milder conditions (0.5 *M*, pH 8.5) (see Fig. 6). In this case, the reactivity of dCMP is negligible compared to that of dTMP, opposite to the effect of HA.

The mutagenic effect of hydroxylamine: We have shown previously[2] that HA exerts two chemically different effects on phage T4 treated *in vitro:* a rapid action which is only lethal and not mutagenic and a slow but highly mutagenic effect. The nonmutagenic effect can be almost completely suppressed by high concentra-

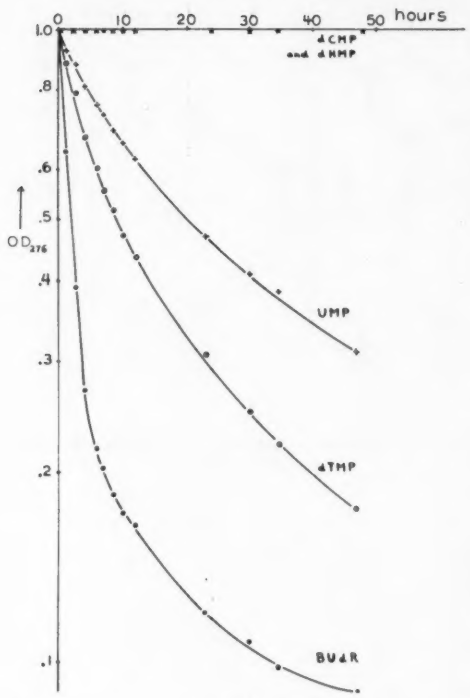

FIG. 6.—Decrease of UV absorption at 276 mμ of various nucleotides in 0.5 *M* hydrazine at pH 8.5.

tions of both NaCl and HA. The induction of forward mutations under such conditions has been examined already.[2]

The induction of reverse mutations is illustrated in Figure 7 for a highly inducible mutant. Reversion induction with HA apparently is a one-hit phenomenon which follows the equation

$$\frac{K}{B} = \frac{K_0}{B_0} + \alpha t = \frac{K_0}{B_0} + \frac{\alpha}{\beta} n.$$

(K or B are the titers of phages on bacteria K or B. K_0 or B_0 are these titers at zero-time treatment. α is the probability of reversion induction per unit time and phage, while α/β is this probability per lethal hit n ($= \ln B_0/B$) and phage.)

Curves of this kind have been examined in more detail in a previous paper[5] for the case of nitrous acid. The mutagenic effect of HA, however, is more pronounced since, owing to HA treatment, the concentration of phages (per ml) that produce plaques on K may increase by as much as a factor of 100.

HA exhibits such a strong effect on BU, as compared to T, that it seemed worth while to examine its action on T4 phages which had been grown in the presence of BUdR. Figure 8 shows that these phages are inactivated by HA much more rapidly than those not containing BU. This is similar to the effect of UV on such phages.[10] The inactivation slope in Figure 8 later levels off, probably because about 5 per cent of the phages in our stock contained little or no BU. The frequency of *r* mutants per viable phage also decreased during the initial part of the HA treatment, i.e. *r*-mutant phages died faster than certain nonmutant ones, and new HA-induced mutants could significantly increase the *r*-mutant frequency only after longer HA treatment. This proves both that the rapidly killed BU-phages contained more *r* mutants than the surviving non-BU-phages and that the reaction of HA with BU in DNA is lethal but little or not at all mutagenic. The same was observed when a BU stock of the *r*II-type mutant N 24 was treated by HA: no increase in the frequency of revertants per viable phage could be observed during the initial rapid inactivation.

The effect of hydrazin: $N_2H_4 \times 2$ HCl acts similarly to $NH_2OH \times$ HCl in that it inactivates phages faster at 0.1 *M* than at 1 *M* concentrations and the effect increases with pH. However, the mutagenic effect of hydrazin on phage T4 at pH

8.3 is much smaller than that of HA. The frequency of forward (r) mutations barely increases, and when the rII mutants AP 275, N29, and N17 were tested for reversion induction with 1 M hydrazin, the respective α/β values were 3, 0.01, and 0.005 in units 10^{-6}, i.e. 10 to 100 times smaller than with HA. Although we cannot exclude the possibility that hydrazin inactivates phages mainly by affecting their protein, the more likely explanation of its small mutagenic effect seems to be that it preferentially attacks thymine in DNA with a predominantly lethal effect.

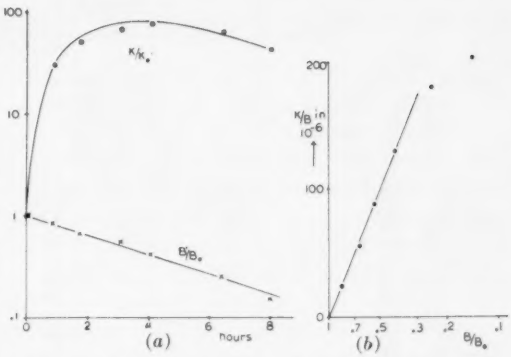

FIG. 7.—Kinetics of the induction of reverse mutations for the highly reversion inducible T4 rII mutant AP 72.
(*a*) Titer of viable phages (B/B_0) and titer of revertants (K/K_0) plotted logarithmically against the time of HA treatment.
(*b*) Frequency of revertants per viable phage (K/B) plotted linearly against the logarithm of the phage titer (B/B_0). For longer treatment, the curve deviates from the straight line, since the relative proportion of mutagenic versus lethal hits decreases.[2]

The specificity of reversion induction: The induction of reverse mutations by HA was examined quantitatively for a number of rII-type mutants which had been induced by different means. For each mutant, the inactivation curve and the induction curve (see Fig. 7*b*) was determined by at least 5 points. The approximate α/β values obtained in this way are summarized in Table 1. It can be seen that certain mutants, induced by 2-aminopurine, display a much larger reversion inducibility by HA than others. (It should be mentioned that for mutants which are highly reversion-inducible by HA the K_0/B_0 values given in Table 1 are slightly higher than the values in the phage stock, since even the short exposure to HA in the cold exerts a slight mutagenic effect.)

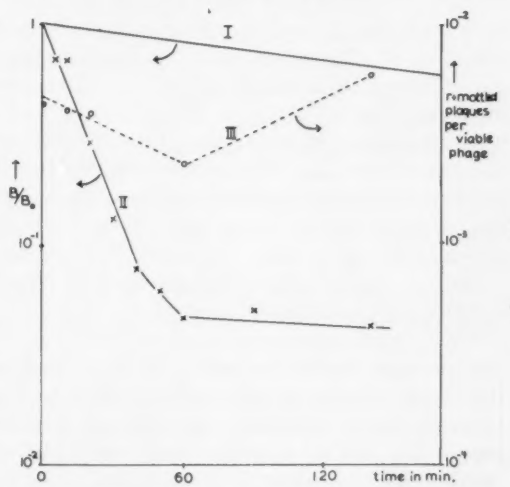

FIG. 8.—Reaction of HA with BU-containing standard type phage T4.
Curve I: Survival of phages not containing BU.
Curve II: Survival of BU-containing phages.
Curve III: Change in the frequency of rII mutants per viable phage for BU-containing standard type phages.

When in previous work the reversion induction by nitrous acid[5] had been analyzed, it was observed that certain mutants could not fully express all of their

TABLE 1

RATES OF REVERSION INDUCTION BY HYDROXYLAMINE*

Mutant	K_0/B_0†	α/β	K_0'/B_0	α'/β
AP 114	4.4	450	70	1040
AP 275	1.2	280	6	240
AP 72	0.7	134	13	158
AP 12	0.7	78	13	194
AP 41	0.3	62	5	265
N 24	0.3	0.2	30	10
N 29	0.05	0.1	5	1
HA 54	0.08	0.1	3	3
AP 61	0.13	0.08	21	18
N 19	0.04	0.06	5	6
HA 45	0.01	0.06	1	4
N 7	0.02	0.04	7	2
HA 11	0.02	0.04	7	8
N 12	0.04	0.03	6	2
N 17	0.02	0.03	—	—
N 31	0.02	0.03	3	6
HA 25	0.003	0.001	0.5	0.1
AP 156	0.008	0.002	5	5
AP 70	0.04	0.01	10	6
AP 83	0.006	0.005	—	—

* All values are given in units 10^{-6}.
† K_0/B_0 = Frequency of revertants per viable phage at zero time treatment. K_0'/B_0 = Same, but with preadsorption on bacteria *B* before plating on *K*. α/β = Frequency of induced revertants per viable phage and per lethal hit. α'/β = Same, but with preadsorption.

induced revertants when plated directly on bacteria K, but they could do so when they were first allowed to multiply in (UV-killed) bacteria B, which then were plated on K. Therefore, again the HA reversion inducibility of all mutants was also examined by preadsorbing the HA-treated phages on bacteria B. The results are given again in Table 1, in the last two columns. It is apparent that for the five highly reversion-inducible mutants the α'/β values obtained with preadsorption are not much larger than the α/β values without preadsorption. For the mutants of low inducibility, the background of spontaneous revertants (K_0'), after preadsorption on B, is so large that we could not measure accurately the α'/β value but can give only an upper limit. It is clear, however, that even with preadsorption none of the other mutants has a high enough α'/β value to be a candidate for the class of highly reversion-inducible mutants.

Some further examination of induced revertants: For a chemical interpretation of the observed mutagenic specificity, it is important to know whether induced revertants arise by genuine backmutations to the standard genotype or whether they are due to suppressor mutations at another site. It has been mentioned previously[1] that no *unspecific* suppressor locus exists such that *each* *r*II mutant could revert by a mutation at this locus. *Specific* suppressor mutations, however, do exist for certain *r*II mutants. They may give rise to phages with different phenotypic properties from the standard type, or they may be detectable only by more refined techniques, i.e., by recombination or mutation tests. E.g., several *r*II mutants produce revertants giving tiny plaques on bacteria K in addition to the normal revertants with wild type plaques. These tiny revertants have not been counted at all in the preceding analysis.

By purely genetic means we cannot prove unequivocally that a revertant arose by a genuine backmutation and does not have a genotype different from that of the standard type phages. But certain results make it at least likely that most revert-

ants of *r*II mutants that produce wild type plaques on both bacteria K and B correspond to genuine backmutations.

The functional property of revertants obtained after HA treatment of phages was examined by replating isolated revertants on both bacteria B and K. Ten to 20 revertants were analyzed in this way for most of the *r*II mutants mentioned in Table 1. Most of them gave wild type plaques on both bacteria. Exceptions were some revertants of AP 114 and all of AP 70 which produce large wild type plaques on B and all induced revertants of AP 12 (or AP 28, which is at the same AP hot spot as AP 12) which gave *r* plaques on B. These exceptions may be due either to a suppressor mutation or to a second mutation present already in some or all phages of the stock. For AP 114, crosses between such revertants and standard type phages gave only the same two types (large wild and wild) and not the original *r* mutant, showing that both explanations are still possible. These exceptions have not been investigated further.

Twenty-three wild type revertants which originated from HA induction of AP 41, 72, 114, and 275 and showed wild type plaques on both bacteria K and B were backcrossed to standard type phages. None of the lysates of a cross showed any significant increase in the frequency of *r*-type plaques above the frequency expected from the background in the stocks. Hence, no evidence for suppressor mutations was observed for these revertants.

In addition to the functional and recombination test, the observed mutagenic specificity argues against the possibility that most *r*II mutants can revert to a wild phenotype by suppressor mutations at several sites, for one would expect the mutagenic specificity of a suppressor mutation in simple base-pair exchanges to be, in general, independent of the specificity of the original mutation. It would then be hard to understand why none of the 5-bromouracil (N)- and HA-induced mutants was as highly inducible to revert as some of the AP mutants.

We conclude that most induced revertants which produce plaques of standard phenotype on both bacteria B and K probably constitute genuine backmutations to the standard genotype.

Discussion.—Both hydroxylamine (HA) and hydrazine react with pyrimidines and not with purines, inverse to the effect of treatment by low pH or ethylating agents. They are very specific chemicals, since they differ in their action on U, T, and C, and HA even separates UR and the various derivatives of C from pseudouridine. HA can be employed to mutate preferentially certain bases or base pairs.

The main mutagenic effect of HA is apparently caused by its reaction with C. Although some reaction with T could also be expected, it must be slow, since so far it has not been detected chemically. The reaction product with C is apparently compound II in Figure 4*b*. The attachment of HA to C opens the resonance structure of the pyrimidine ring and destroys the normal pyrimidine absorption spectrum. Compound II is stable for several days at neutral pH but on acidification is converted into compound III. We do not know whether or not this conversion occurs already at neutral pH when the HA-treated phage DNA enters the bacterium. But whichever compound may be present, the HA-reacted C should no longer be able to pair with G since the 3-position hydrogen interferes sterically with the hydrogen atom on G. However, this compound can make at least one hydrogen bond with A and thus cause its incorporation in the place of G. HA is small and

far enough out of the pyrimidine plane that it should not sterically hinder the pairing between A and compound II. Whatever the detailed chemical events may be it seems clear that HA predominantly induces the base pair transitions

$$\begin{matrix} C & T \\ G & A \end{matrix} \qquad (1)$$

Corresponding to this chemical directional effect, we found that transition mutants fall into two classes with respect to the induction of reverse mutations by HA. Five mutants that had been induced by AP are highly inducible by HA to revert. Most revertants of four of these mutants could not be distinguished from standard type phages by functional or recombinational tests. It seems likely, therefore, that these mutants carry a G-HMC pair at their mutant site. The fifth mutant, AP 12, yields only revertants with *r* plaques on bacteria B; it is still open whether these revertants arise by a suppressor mutation or whether they already contain a second *r* mutation at some other site. These five AP mutants are also especially reversion-inducible by nitrous acid (after preadsorption on B),[5] by ethylethane sulfonate,[11] and by low pH treatment.[12] Thus, both the chemical and the mutagenic observations agree that all these agents induce transitions preferentially in the direction shown in equation (1). This directional effect is the same irrespective of whether G or C is attacked by the agent.

The directional effect of HA is much stronger than for any of the other agents, for the difference in induction rates, α/β, between highly and slightly or not inducible mutants is larger than 100. It seems likely, therefore, that these lowly or noninducible mutants have an A-T pair at their mutant site unless few of them are caused by an exceptional DNA change. Three mutants (AP 70, 83, 156) that respond little to HA, nitrous acid, or low pH gave a stronger response to ethylethane sulfonate.[11, 12] This indicates some higher mutagenic specificity of the corresponding DNA sites with respect to the various mutagens and shows that the mutagenic direction can be safely deduced only when mutagens are employed that have an especially high preference for one base.

When HA is compared to other mutagens, its lethal effect is very small in relation to its mutagenic effect. This follows from the large frequency of forward mutations per lethal hit[2] as well as from the large α/β value of about 10^{-4} for the highly reversion-inducible mutants. Since there are about 7×10^4 HMC bases in a T4 phage (assumed to be the same as measured for phage T2[13, 14]), the observed probability, α/β, of reversion induction per lethal hit is about that expected if a reversion is induced by the HA action on a *certain* HMC base (or few of them) while the action on most other HMC bases is lethal. This agrees with our picture that the mutations concerned here are due to the alteration of a single nucleotide pair in DNA. Furthermore, it seems probable that HA exerts its lethal effect on DNA mostly by the same mechanism which at another site is mutagenic. This is in contrast, e.g., to nitrous acid, whose effect on G is not mutagenic but apparently lethal.[15]

It is worth-while to compare the data on reversion induction with those on spontaneous reversion indices,[1] for transition mutants that are highly inducible to revert by HA or the other agents mentioned before also revert spontaneously with especially high frequency. This suggests that, at least in phage T4, spontaneous transitions occur more frequently in the direction of equation (1) than in the opposite

direction. Similar observations have been made for spontaneous transversions.[12]

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*COMPLEX UNILATERAL HYBRIDIZATION IN LYCOPERSICON HIRSUTUM**

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Unilateral hybridization, as originally defined by Harrison and Darby,[1] is the restriction of hybridizing between two species to one of the reciprocal crosses. This behavior was first encountered in attempts to cross self-fertile species to those with multiple allelic self-incompatibility. Often such crosses succeed only when the self-fertile parent is used as female. The observation that failure of such crosses was due to pollen tube growth stoppage similar to that of the self-incompatibility reaction led to the conclusion that the two phenomena, self-incompatibility and unilateral hybridization, were controlled by the same genetic system. These suppositions seemed to be verified by the discovery of unilateral hybridization between two self-fertile species, *Antirrhinum orontium* and *A. majus*.[1] The latter species is thought to have become self-fertile during historic times through man's selection, but appears to have retained that portion of the incompatibility system responsible for unilateral hybridization.

Lewis and Crowe[2] consider a species such as *A. majus* an evolutionary intermediate between self-incompatibility and true self-fertility. In numerous cross pollinations of a wide variety of self-fertile species and related species with multiple allelic self-incompatibility, styles were examined after pollination for pollen tube growth. In almost every case, pollen tube growth was inhibited when pollen from self-fertile plants was placed on the styles of self-incompatible plants. In the reciprocal crosses pollen tubes grew normally. In general, cross pollinations be-

tween either two self-fertile species or two self-incompatible species were characterized by no pollen tube inhibition. Four anomalous self-fertile species differed from the rest of the self-fertile group in that their crossing relationships were identical to those of the self-incompatible species. A consideration of the history and morphology of these species led to the conclusion that they have recently been derived from self-incompatible progenitors. A scheme is presented in which such evolution could occur through mutation of the *S* genes responsible for the specific properties of the incompatibility system accompanied by mutations of other, modifying genes. The eventual product of such an evolutionary sequence is thought to be the typical, self-fertile species. Thus, species have been considered to be of two types with respect to unilateral hybridization: first, typical self-fertile species; and secondly, self-incompatible and intermediate self-fertile types showing unilateral hybridization with those typically self-fertile.

An investigation of the inheritance of self-incompatibility and unilateral hybridization was undertaken in the green-fruited tomato, *Lycopersicon hirsutum*, in order to determine more precisely the relationships between the two phenomena. *L. hirsutum* is known to include both self-fertile and self-incompatible forms which hybridize fairly readily not only among themselves but also with the garden tomato, *L. esculentum*.[3] It was thought that in crosses of closely related forms differing with respect to unilateral behavior or self-incompatibility, the problem could be investigated with the least complication by other genetic differences.

Four separately collected accessions of *L. hirsutum* were included in a preliminary study of pollination relationships within the species. Three of the collections represent the botanical form *typicum*, characterized in part by large, dark yellow flowers, whereas the fourth is form *glabratum*, with smaller, less yellow flowers. The four collections were in no way intended to represent a cross section of the variability of the species but happened to be the materials on hand at the onset of the investigation. Each possible cross was made on six or more flowers of each plant and on two or more plants of each accession, except for the Chillon I accession (*f. typicum*), represented by a single, clonally multipled plant. All crosses that failed were repeated on a similar scale, and, in addition, styles were examined using the method of Martin[4] to determine the extent of germination of pollen and growth of pollen tubes. Finally, all possible crosses of the four collections to *L. esculentum* were tried.

The crossing results were clear and definite (Table 1). In most cases, if a cross

TABLE 1

CROSSING COMPATIBILITIES OF FOUR COLLECTIONS OF *Lycopersicon hirsutum* AND OF *L. esculentum**

	Male Parents				
	L. hirsutum				*L. esculentum*
Female Parents	*f. typicum* Chillon I	*f. typicum* Cajamarca	*f. typicum* Chillon II	*f. glabratum* Baños	
Chillon I	Self-incompatible	0	0	0	0
Cajamarca	x	Self-incompatible	0	0	0
Chillon II	x	x	Self-fertile	0	0
Baños	x	x	x	Self-fertile	0
L. esculentum	x	x	x	x	Self-fertile

* Successful crosses are indicated by an x, and unsuccessful crosses are marked 0.

was successful, all of the flowers pollinated set fruit. Examinations of styles showed that all crosses that failed were characterized by pollen tube growth stoppage similar in appearance to that of the self-incompatibility reaction. However, the length of pollen tubes before inhibition varied in the different unsuccessful crosses.

The crossing results contrast in several respects with those reviewed by Lewis and Crowe.[2] First, unilateral hybridization occurs between the two self-incompatible forms, Chillon I and Cajamarca, a situation neither anticipated nor previously described. Secondly, crossing behavior is of more than two kinds. In fact, each collection demonstrates a unique response in pattern of crosses to the other four. However, the patterns are not haphazard. The five entities are arranged along the top of the table in such order that each entity will cross as male parent only to the entities to the right and as female only to the entities to the left. Thirdly, the occurrence of three levels of unilateral behavior among self-fertile forms, as found here, has not been previously demonstrated and cannot be readily reconciled with the Lewis and Crowe[2] model of the evolution of self-fertility from self-incompatibility.

It is apparent from these results that unilateral crossing behavior is more complex, at least in *L. hirsutum*, than has been suspected. The relationship of this behavior to the genetic system controlling self-incompatibility is not clear-cut. The genetics and the physiology of the two systems badly need study, and this can be done best in species such as *L. hirsutum* where closely related forms show drastically different behavior.

Summary.—Unilateral hybridization occurs among *L. hirsutum* lines and between these and lines of *L. esculentum*. The restriction of crosses follows an orderly pattern not previously found in the plant kingdom and not in accordance with present theories of the origin and evolution of self-incompatibility and self-fertility.

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*CHROMOSOME BREAKAGE ACCOMPANYING GENETIC RECOMBINATION IN BACTERIOPHAGE**,†

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Genetic recombination in bacteriophage was first observed by Delbrück and Bailey[1] and by Hershey and Rotman,[2] who showed that cells simultaneously infected with two different mutants of phage T2 yield, in addition to the two infecting types, both doubly mutant and wild type progeny phages. Subsequently, ex-

tensive studies have been made of bacteriophage recombination, especially with the phages T1, T2, T4, and λ. Hundreds of mutants have been crossed, and for each phage type the observed recombination frequencies may be represented on a single unbranched genetic map.[3-6]

The genetic material of these bacteriophages is known to be DNA and it is commonly assumed that the genetic information resides in the linear sequence of nucleotides which comprise the DNA molecule. Under this assumption, genetic recombination in bacteriophage corresponds to the production of a nucleotide sequence derived partly from one parental line and partly from another. This may be imagined to occur in either of two ways. (1) *Copy choice:* The recombinant sequence is synthesized *de novo* by copying first one parental sequence, then another. (2) *Breakage:* The recombinant sequence is formed by the association of DNA fragments from different parental lines.

Recombinant phages produced by copy choice would be free of parental DNA, whereas breakage could result in the appearance of portions of parental DNA in recombinant phages.

In this paper, we describe experiments with the bacteriophage λ designed to see whether there is parental DNA in recombinant phages. Two-factor crosses were made between λ and λ heavily labeled with the isotopes C^{13} and N^{15}. Then density-gradient centrifugation[7, 8] was used to determine the distribution of labeled parental DNA among both parental and recombinant genotypes of the progeny. Our results demonstrate the presence of discrete portions of parental DNA in recombinant phages.

Materials and Methods.—Preparation of $C^{13}N^{15}$ media: Algal hydrolysates: Carbon-13 of 93% isotopic purity supplied in the form of methane[9] was oxidized to CO_2 by repeated passage over CuO at 850°C. The CO_2 was absorbed in saturated $Ba(OH)_2$ solution. A 6.8 gram portion of the resulting $BaC^{13}O_3$ was used along with $N^{15}H_4Cl$ of 99% isotopic purity[10] for the growth of *Ankistrodesmus* following the procedure of Davern.[11] After harvest by centrifugation, the algae were suspended in 15 ml of *N* HCl and kept at 100° for 6 hours. The mixture was centrifuged to yield a supernatant called H-1 and a pellet which was resuspended in 15 ml of 6 *N* HCl and refluxed for 20 hr. After cooling, the mixture was centrifuged to yield the supernatant H-2 and a pellet which was discarded. Hydrolysates H-1 and H-2 were separately evaporated to dryness *in vacuo* at room temperature and each residue was taken up in 5 ml of water. After three repetitions of this process, the final solutions were brought to pH 6 by the addition of carbonate-free NaOH solution. To H-2 was added 50 mg of HCl-washed decolorizing charcoal, and the mixture was shaken for 1 hr. Then both H-1 and H-2 were filtered through fine sintered-glass disks and sterilized by passage through type HA millipore filters. Both H-1 and H-2 were made up to 10 ml with sterile water and kept frozen until used.

Yeast extract: The hydrolysate H-1 was used to support the growth of yeast[12] for approximately ten generations in a medium of the following composition: $N^{15}H_4Cl$, 0.10 gm; Hydrolysate H1, 10 ml; NaCl, 0.4 gm; K_2HPO_4, 0.075 gm; $MgSO_4$, 0.30 gm; $CaCl_2$, 0.016 gm; $FeCl_3$, 7×10^{-5} gm; H_2O, 150 ml.

The culture was gently aerated at 30°C until the yeast attained a concentration of 4×10^7/ml and had just stopped budding. After harvest by centrifugation, the yeast was suspended in 5 ml of H_2O and brought to 100°C for 1 min and kept at 37°C overnight. The suspension was then centrifuged and the supernatant, called yeast infusion, was frozen. The pellet was suspended in 10 ml of 6 *N* HCl and refluxed 20 hours, after which the hydrolysate was repeatedly evaporated and then adjusted to pH 6 as described above. Finally, the hydrolysate was added to the yeast infusion, and the combined solution was filtered through a fine sintered-glass disk and sterilized by passage through a type HA millipore filter. This yeast extract was made up to 10 ml with sterile water and was kept frozen.

The isotopic purity of the $C^{13}N^{15}$ media was verified by measurements of the buoyant density of DNA prepared from *Escherichia coli* grown for many generations in a medium containing $N^{15}H_4Cl$ and either H2 or $C^{13}N^{15}$ yeast extract as described below. In both cases, the $C^{13}N^{15}$ DNA was found to be 0.047 gm cm^{-3} more dense than unlabeled DNA from the same organism. The calculated increment for the Cs salt of *E. coli* DNA with 99% N^{15} and 93% C^{13} is 0.050 gm cm^{-3} in the absence of selective solvation.

Bacteriophages: Phage λ++ is the "wild type" of Kaiser.[13] Phage λ*h* was derived from λ++ and has extended host range enabling it to plate on CR63. Phage λ*cmib*$_2^+$ is described by Kellenberger *et al.*[14] and is designated here as λ*cmi*.

Bacteria: Strain 3110, a λ-sensitive derivative of *E. coli* K12, was used for the preparation of λ*cmi* stocks and as host for crosses. Strain C 600[15] was used for plating as was the λ-resistant strain CR63.[16] Strain K12S made lysogenic for λ++ was used for the production of λ++ stocks by induction.

Media: Tryptone broth and suspension medium were prepared according to Weigle *et al.*,[8] and A medium is 7 gm Na_2HPO_4, 3 gm KH_2PO_4, 0.6 gm $MgSO_4$, 0.5 gm NaCl, 5×10^{-4} gm $FeCl_3$, and 1 liter H_2O, to which carbon and nitrogen sources were added separately.

Phage stocks: Phage λ++ was prepared by UV induction of K(λ) grown in A medium containing either 1 mg/ml $N^{15}H_4Cl$ and 0.05 ml/ml each of H-2 and yeast extract or 1 mg/ml $N^{14}H_4Cl$, 3 mg/ml charcoal-treated casamino acids and 1 mg/ml Difco yeast extract. The bacteria were grown approximately 7 generations to 2×10^8/ml, centrifuged, suspended at 10^8/ml in A medium and irradiated. Carbon and nitrogen sources were then added as above and the induced culture was aerated for 100 minutes, after which the culture was lysed by saturation with chloroform. The burst size was approximately 100 for both λ$C^{13}N^{15}$ and $C^{12}N^{14}$ λ++. The lysates were centrifuged at 50,000 × *g* for 120 minutes to sediment the phage. The pellet was resuspended by standing overnight in suspension medium.

Phage λ*cmi* was prepared by one cycle of lytic multiplication in 3110 grown in A medium with either $C^{13}N^{15}$ or $C^{12}N^{14}$ as above. The bacteria were grown approximately 8 generations to 5×10^8/ml and λ*cmi* in suspension medium was added to a multiplicity of 3. Ten minutes after infection, a 3-fold dilution with fresh medium was made and aeration was continued for 130 minutes. The culture was then lysed by saturation with $CHCl_3$. For the preparation of the λ*cmi* stocks used in cross II, the culture was made 0.003 *M* in ethylene diamine tetra-acetate, and 0.1% in lysozyme 1 min before the addition of $CHCl_3$. The burst size was approximately 60 for both labeled and unlabeled λ*cmi*. The phages were purified by low- and high-speed centrifugation and suspended in suspension medium.

The frequency of all morphology mutants was below 0.01% in the stocks used in these experiments.

Density-gradient centrifugation: The technique of preparative density-gradient centrifugation described by Weigle *et al.*[8] was employed with several modifications. Centrifugation was at 23,000 rpm for 24 hours at 24°C. The cesium chloride solution was overlayered with mineral oil to fill the lusteroid tube in order to prevent its collapse. The initial density was chosen so that all bands formed in the lower half of the gradient. This step, recommended to us by Dr. F. Stahl, prevents the occurrence of an artifact of preparative density-gradient centrifugation which previously has caused us much difficulty. When bands formed in the upper part of the gradient are collected, one invariably finds a satellite preceding the main band. The satellite contains approximately 1% as much material as the main band and precedes it by as many as 6 drops. Phage λ*h* was added in all centrifugations in order to serve as a density marker and to establish, by its characteristic narrow unimodal distribution, that no artifacts due to deceleration or drop collection had disturbed the distribution of the various other phage types.

In all experiments, the recovery of each genotype from the CsCl gradient was essentially complete.

Results.—Two crosses were performed with labeled phages: cross I, λ ++ $C^{13}N^{15}$ × λ*cmi* and cross II, λ ++ × λ*cmi* C^{13}/N^{15}. In both cases, parallel crosses were made using unlabeled parents. These are referred to as dummy crosses I and II. The multiplicities of infection and yields for the various crosses are recorded in Table 1. The dummy crosses were made at approximately the same multiplicities

TABLE 1

Crosses	Multiplicities ++	Multiplicities *cmi*	Burst sizes ++	Burst sizes *cmi*	Recombinant frequencies (%) *c*	Recombinant frequencies (%) *mi*
I λ++ $C^{13}N^{15}$ × *cmi*	2	3	19	46	0.88	0.56
II λ++ × *cmi* $C^{13}N^{15}$	5	2.2	33	12	0.74	0.46

and gave comparable yields.

Dummy crosses: As may be seen in Figure 1, λ*h* and both parental and recom-

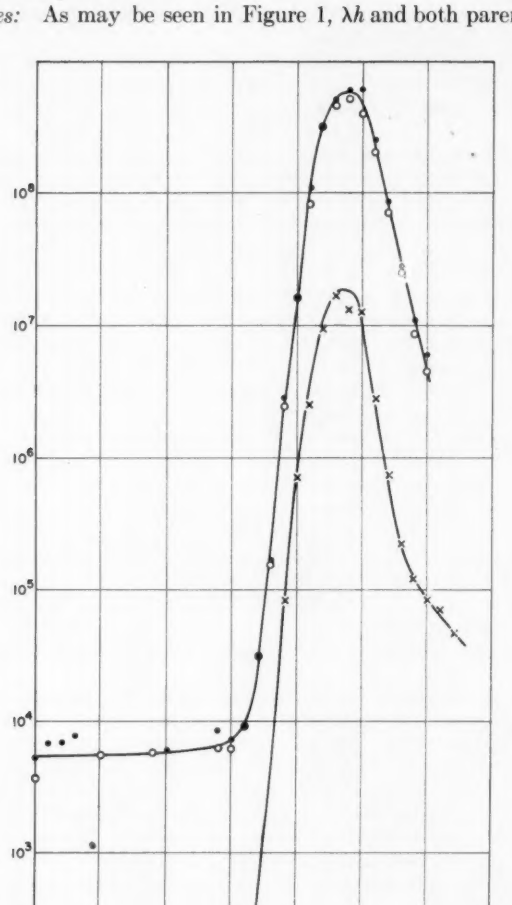

FIG. 1.—Cross λ*cmi* × λ++. Titer of the progeny phages of different genotypes in the drops collected after centrifugation. —●—●—, *cmi*; —○—○—, ++; —x—x—, *h*. λ*h* was added as density and band shape reference. The titers of the recombinants λ + *mi* and λ*c*+ follow exactly the curve of λ*h*, and the points representing the measurements have been omitted to avoid crowding the picture.

binant types emerging from a dummy cross exhibit the same narrow unimodal distribution in the density gradient. This finding allows us to attribute any deviation of the distributions found in crosses I and II from the distribution of the λ*h* control to the presence of the density labels C^{13} and N^{15}.

Heavy parental stocks: The density distributions of the $C^{13}N^{15}\lambda$ ++ stock used in cross I and of the $C^{13}N^{15}\lambda cmi$ stock used in cross II are shown in Figures 2 and 3.

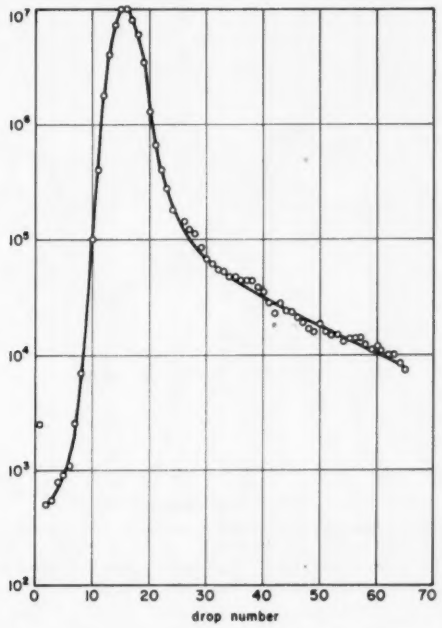

FIG. 2.—Density distribution of $C^{13}N^{15}$ λ++ used in cross I. The λ++ phages were induced from a lysogenic culture which had grown for many generations in heavy medium. The peak of the $C^{12}N^{14}$ λ*h*, not shown in the figure, came at drop 78.

The distribution of the heavy λ++ stock is essentially the same as that of λ *h* displaced to higher density by 0.05 gm cm^{-3}. This high uniformity of labeling is to be expected of the heavy λ++ phages, for they were induced from a culture which had grown many generations in heavy medium.

Because the heavy λ*cmi* stock was prepared by only one cycle of growth in uniformly labeled cells infected by unlabeled phages, its density distribution provides information regarding the distribution of parental carbon and nitrogen among progeny phages. An important feature of the λ*cmi* distribution shown in Figure 3 is the existence of two modes in addition to the main component which is fully labeled. According to their position in the density gradient, these two modes correspond to phages with 56 per cent and 78 per cent heavy label. The light parental atoms are presumably harbored in their DNA and not in their protein, which is not injected upon phage infection.[17] If all of the DNA were parental and all the protein new, it may be estimated from the composition of λ and the

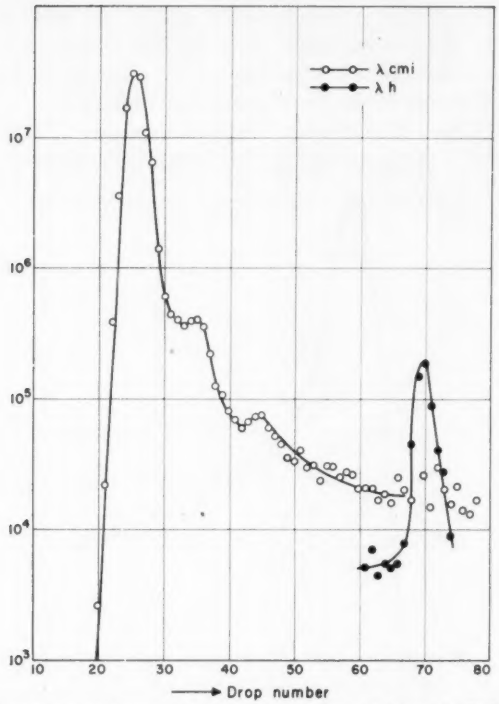

FIG. 3.—Transfer experiment with λ*cmi*. λ*cmi* grown in bacteria $C^{12}N^{14}$ were used to infect bacteria $C^{13}N^{15}$ in a $C^{13}N^{15}$ medium. After one cycle of growth, the λ*cmi* labeled with $C^{13}N^{15}$ were centrifuged. The figure gives the titers of the phages in the different drops collected after centrifugation. Phage λ*h*$C^{12}N^{14}$ was added as a density reference.

purity of the isotopes used that the phage would contain 54 per cent heavy label. We therefore presume that the phages with 56 per cent heavy label are composed of new (heavy) protein and parental (light) DNA and that the phages with 78 per cent label are composed of new protein and DNA which is half-parental. We shall refer to phages with only parental DNA as "conserved" and to those with half-parental DNA as "semiconserved." Semiconserved phages have appeared in each of the nine experiments in which we have infected labeled cells with unlabeled phages or *vice versa*. However, only when the multiplicity of infection was high have conserved phages also appeared. The yield of infectious hybrid phages has ranged between 1 and 4 per cent of the progeny phages while yields of infectious conserved phage have been found as high as 1 per cent (when $m = 7$) and less than 0.001 per cent (when $m = 0.1$).

Crosses: As may be seen in Figure 4, the density distributions of the progeny of cross I exhibit the following principal features:

(1) The distribution of the originally labeled parental genotype λ++ resembles that found when labeled phages alone are allowed to multiply in unlabeled cells. That is, modes of conserved and semiconserved phages are found in addition to unabsorbed and to essentially unlabeled phages.

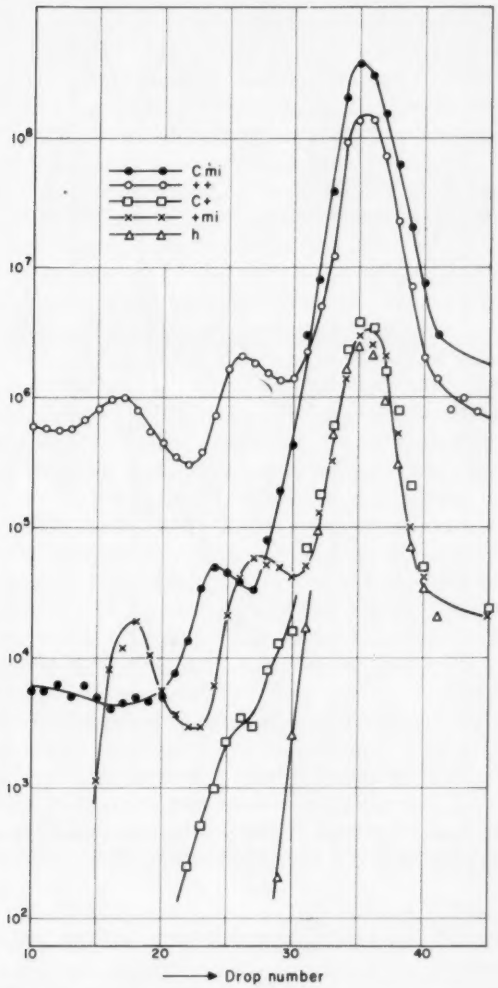

FIG. 4.—Cross λ++ $C^{13}N^{15}$ × λ*cmi* $C^{12}N^{14}$. Titer of the progeny phages of different genotypes in the drops collected after centrifugation. λ*h* was added as density and band shape reference.

(2) The originally unlabeled parental genotype *cmi* emerges from cross I with a very different distribution of label from that of the type ++. Neither conserved nor semiconserved modes are found. The λ*cmi* is essentially unlabeled but with a definite skewness toward higher density.

(3) The recombinant type +*mi* and *c*+ are distributed in strikingly different fashion from each other. The type +*mi* is found in three modes, one essentially unlabeled, one with slightly less label than the semiconserved λ++, and another with slightly less label than conserved λ++. The reciprocal type *c*+ is found

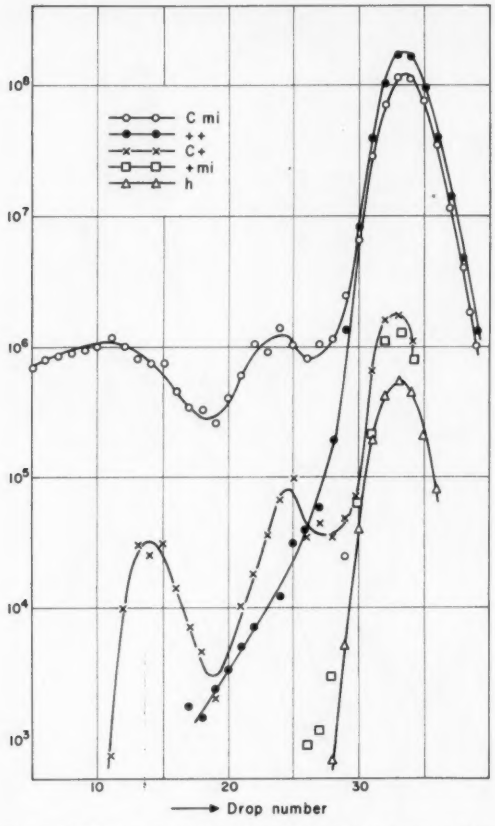

FIG. 5.—Cross λ*cmi* $C^{13}N^{15}$ × λ++ $C^{12}N^{14}$. Titer of the progeny phages of different genotypes in the drops collected after centrifugation. λ*h* was added as density and band shape reference.

in a single band which has a shoulder at a density between that of semiconserved and unlabeled phage.

To insure that the nonequivalent distributions of the two recombinant types from cross I were not due to peculiarities associated with the particular recombinant genotypes, a cross (cross II) was performed with $C^{13}N^{15}$ label in λ *cmi* rather than in λ++. As may be seen by comparison of Figures 4 and 5, the result of reversing the parental label is that the distributions of the two recombinants as well as the two parental types are interchanged.

Discussion.—Replication of the lambda chromosome: The maximum recombination frequency observed[6] for phage λ is only 15 per cent. This circumstance suggests that we may understand the main features of the distribution of parental DNA among progeny phages without at first considering the effects of genetic recombination. In summary, these features are:

(1) Among progeny from infections at high multiplicity, the original parental

DNA is distributed in two discrete modes corresponding to phages with DNA that is entirely original parental (conserved phages) and to phages with half original parental, half newly synthesized DNA (semiconserved phages).

(2) Among progeny from infections at multiplicity of much less than one phage per bacterium, no conserved phages are found; only semiconserved phages appear besides those with DNA entirely newly synthesized.

(3) From crosses between isotopically labeled and unlabeled phages, the genotype of the labeled parent emerges in both conserved and semiconserved modes, but these modes are essentially free of other genotypes.

The finding of semiconserved phages indicates that the DNA complement of λ is equally divided between two subunits which may separate from one another and appear in progeny along with newly synthesized subunits.

The finding of conserved phages at high but not at low multiplicities of infection may be explained in two ways. Either the two parental subunits never came completely apart or else they did so only to become reassociated by chance in the same progeny particle. The latter alternative is, however, made improbable by the finding that semi-conserved phages emerging from crosses between labeled and unlabeled parents are almost entirely of the genotype that was originally labeled. The substantial yield of labeled recombinants prevents us from ascribing this result to very poor mixing of vegetative phage in the early pool. In view of this, the hypothesis of separation and reassociation would require that the genes *c* and *mi* be restricted to only one of the two subunits and that the other never appear in progeny if the two became separated. This seems very unlikely. Accordingly we shall consider the first alternative only, in which the two parental subunits found in conserved phages never came apart.

Thus the DNA complement of λ is a single structure capable of remaining intact throughout the processes of infection and maturation. This does not imply that this structure replicates conservatively. On the contrary, the finding of conserved phages only at high multiplicities of infection suggests that the conserved phage may never have replicated; they may be "nonparticipating" phages. We are led to conclude that *the entire DNA complement of λ is a single semiconservatively replicating structure.*

In this respect, the DNA of λ behaves as a single molecule possessing the Watson-Crick structure replicating according to the scheme proposed by them.

In the remainder of this discussion, we shall refer to the DNA complement of λ as its chromosome.

The mechanism of recombination: Our main finding relevant to the mechanism of genetic recombination is that discrete amounts of original parental DNA appear in recombinant phages. This suggests that *recombination occurs by breakage of parental chromosomes followed by the reconstruction of genetically complete chromosomes from the fragments.*

Consideration of the detailed distribution of parental DNA among recombinants leads to several additional conclusions regarding the recombination process. The finding of a distinct recombinant class containing substantially more than 50 per cent original parental DNA indicates that *recombination by chromosome breakage may occur without separation of the two subunits of the parental chromosome.* If, as has been argued above, replication does not occur without separation of chromo-

somal subunits, it may also be concluded that *chromosomes need not replicate in order to recombine.*

From the positions of *c* and *mi* on the genetic map of λ (Fig. 6), it is seen that a break between these loci leaves an 85 per cent fragment of the map attached to the

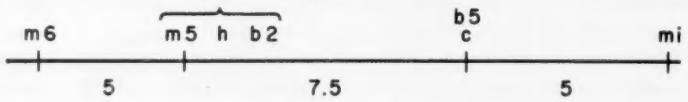

FIG. 6.—Genetic map of λ.

c locus and 15 per cent to the *mi* locus. In both crosses I and II, there occurs a mode of recombinants with approximately 86 per cent labeled parental DNA, and these recombinants carry at the *c* locus the allele contributed by the labeled parent. Thus it seems that *the probability of recombination between two loci is at least approximately proportional to the amount of DNA between them.* The recombinant type which appears with 90 per cent labeled DNA is also found in another mode with approximately 45 per cent labeled DNA. This class of recombinants could have been derived by semiconservative replication from recombinant chromosomes of the type carrying 90 per cent label or they could have been produced by breakage of hybrid chromosomes of parental genotype. It seems reasonable to suppose that both processes contribute recombinants to the mode at 45 per cent. Finally, we might expect the recombinant bearing the *c* allele brought in by the unlabeled parent to be distributed in two labeled modes at approximately 14 per cent and 7 per cent. However, phages so slightly labeled could not be resolved from the large band of unlabeled phage. Only a shoulder on the dense side of the unlabeled peak would be expected. As may be seen in Figures 4 and 5, such a shoulder is in fact observed.

The findings of this experiment thus may be understood in terms of a recombination mechanism involving breakage of a semiconservatively replicating two-stranded chromosome. The fragments produced could become incorporated into complete chromosomes either by joining of appropriate fragments (break and join) or by the completion of a fragment by copying the missing region from the homologous portion of a chromosome of different parentage (break and copy). Figures 7 and 8 show structurally plausible models for recombination by these two processes. The present experiments do not distinguish between these alternatives. Neither do they rule out the possibility of some copy choice recombination.

The contribution of original parental DNA to progeny bearing the initially unlabeled genotype does not conflict with our main conclusion that recombination occurs by breakage. However this distribution is not in simple accord with the notion that the probability of recombination between loci is always proportional to the amount of DNA separating them on the chromosome. The existence of favored breaking places as well as several other complexities, either experimental or genetic, may be invoked to explain the distribution of λ*cmi* in cross I or of λ++ in cross II. However we prefer to postpone discussion of this matter until it has been investigated further. Our conclusions are strongly supported by the experiments reported in the next paper,[18] which shows that in two-factor crosses between P^{32}-labeled and unlabeled λ, more P^{32} is transferred to recombinant progeny

MODELS FOR RECOMBINATION BY BREAKAGE OF DNA MOLECULES

Solid and dashed lines represent single chains with nucleotide sequences descended from different parents.

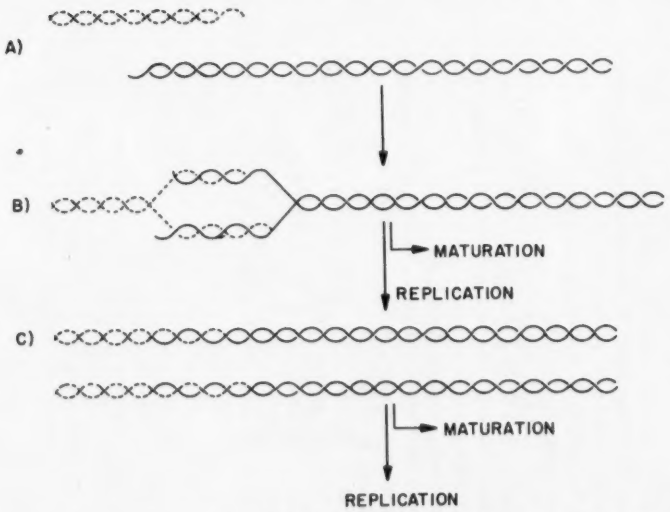

FIG. 7.—*Break and Join.* (*A*) Fragments from different parental lines possessing a region of common nucleotide sequence. (*B*) Association of fragments by complementary base-pairing in the region of common sequence. The recombinants with 90% parental DNA observed in the present experiments would contain chromosomes resembling this structure. (*C*) The two replication products of (*B*). Some of the recombinants with 45% parental DNA would have this type of structure.

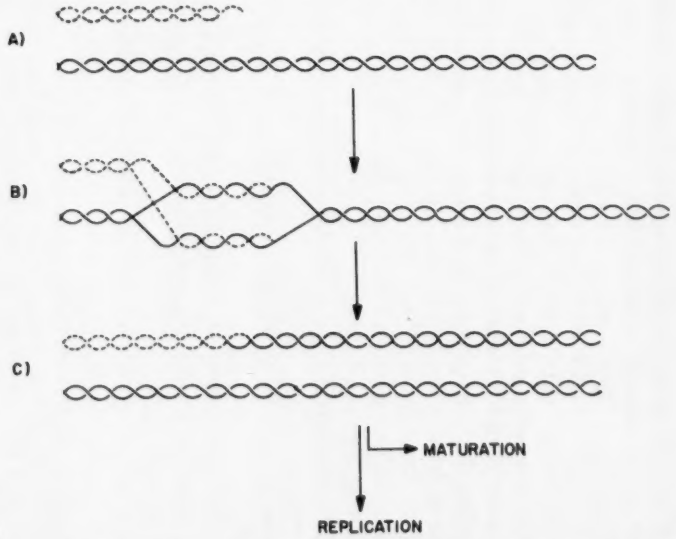

FIG. 8.—*Break and Copy.* (*A*) An intact molecule and a fragment from different parental lines. (*B*) Association by complementary base-pairing over a region of common nucleotide sequence. (*C*) The fragment is restored by copying the intact molecule. The recombinants found with 90% parental DNA would correspond to the restored fragment.

than to progeny with the genotype of the unlabeled parent. Although the P^{32} experiments give no information on the subunits of the DNA, they do give the integrated total amount of the original parental DNA transferred to the recombinants. This value also seems to show that distances along the genetic map are proportional to the amounts of DNA in the phage chromosomes.

Summary.—We have performed experiments designed to detect original parental DNA in recombinant phages. Density-gradient centrifugation was used to determine the distribution of labeled parental DNA among both parent and recombinant genotypes emerging from a two-factor cross between unlabeled phage λ and λ heavily labeled with the isotopes C^{13} and N^{15}. Our results demonstrate the presence of preferred quantities of original parental DNA in recombinant phages and support the following main conclusions:

(1) The DNA complement of λ is contained in a single semiconservatively replicating chromosome.

(2) Recombination occurs by chromosome breakage, although other mechanisms are not excluded.

(3) Both chromosomal subunits are broken during recombination.

(4) Distances along the genetic map are proportional to the amounts of DNA in the phage chromosome.

(5) The phage chromosome need not replicate in order to undergo recombination by breakage.

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† Aided by grants from the National Foundation and the National Institutes of Health.

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EXCHANGE OF DNA IN THE RECOMBINATION OF BACTERIOPHAGE λ

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Genetic recombination in λ at the molecular level studied in the preceding paper[1] can also be studied by means of the following experimental system:

Mutants of λ are known[2] which differ among themselves and differ from the wild type by their densities. Density-gradient centrifugation permits the physical separation of phages of different densities. For two well-studied mutants at least, it was found that the differences in densities with respect to the wild type are due to genetic markers which can be localized on the genetic map of λ. These density markers behave in crosses as does any other marker. Thus we have described the marker *b2* in the region *m5–h* of the linkage group of λ, and in the present work we shall also make use of another character called *b5*, situated in the *c* region. No phenotypic mixing occurs with these markers, that is to say that the density of the progeny phages, in a cross involving *b2* and *b5*, corresponds strictly to their genotype.

In a cross the density differences are additive (within the experimental error) for the recombinants. In other words, a cross $b2b5^+ \times b2^+b5$ (both phages are less dense than the wild type) yields recombinants of wild type density $b2^+b5^+$ and recombinants *b2b5* less dense than either of the parents. When the progeny of such a cross is centrifuged in a density gradient, four bands appear, two of which correspond to the parental densities and two new ones containing only recombinants.

We have made use of this system to see if there was a physical exchange of DNA in genetic recombination. We labeled the DNA of one of the infecting parents with P^{32} and measured the distribution of P^{32} among the progeny. The presence of P^{32} in phages different in density from the labeled parent, particularly in the recombinant phages, shows that recombination involves a physical exchange of DNA. These experiments also show that breakdown and reutilization of the parental infecting DNA is probably negligible.

Material and Methods.—Media: Difco Tryptone 1% with 0.5% NaCl; *M* 9 to which 0.5% of Difco amino acids was added, called here *M* 9 AA; Difco Neopeptone 1% with 0.5% NaCl; and adsorption medium, 0.01 *N* $MgSO_4$ in distilled water.

Bacterial strains: W3110 (Lederberg) C 600 (Appleyard[3]) and derivatives of W3110 made lysogenic for λ wild type and λ *b5*.

Phages: $b2^+b5^+$ "wild type" (Kaiser[4]) having a buoyant density in CsCl of 1.508 gm cm^{-3}
b2b5$^+$ (1.491 gm cm^{-3})
$b2^+b5$ (1.501 gm cm^{-3})
b2b5 (1.483 gm cm^{-3})

The mutation *b2* has been described elsewhere.[2] The phage λ*b5* was found in a stock of λ wild type. The original λ*b5* mutant carried another marker besides *b5*, giving its plaques a small size; this morphological marker was eliminated by crossing the mutant with λ wild type. The marker *b5* is in the region *c* and confers on the phage carrying it an immunity different from that of λ. Thus λ*b5* forms plaques on bacteria lysogenic for $\lambda b5^+$ and inversely $\lambda b5^+$ forms plaques on bacteria lysogenic for λ*b5*. We have also shown that (1) λ*b5* crosses with $\lambda b5^+$, (2) it carries the

marker $b2^+$, (3) it has the same host range as λ, and (4) it is located on the bacterial chromosome at the λ site, or very close to it.

Clear mutants of λ*b*5 can be isolated and one of them, *b*5*c*, was used in the crosses to be described here. A clear mutant,[5] c_1 of $\lambda b5^+$ and the morphological marker *mi* were also used. The positions of these different markers on the genetic map of λ are given in Figure 1.

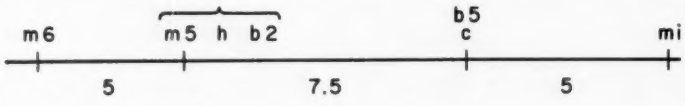

FIG. 1.—Genetic map of phage λ.

The phage λ*b*2*b*5 was obtained in crosses of $\lambda b2b5^+$ with $\lambda b2^+b5$.

Preparation of phage stocks marked with P³²: Bacteria W3110 were grown in neopeptone medium to a density of 2×10^9 cells/ml. The bacteria were infected with phage at a multiplicity of about 0.01 phage per bacterium. After allowing 10 min for adsorption, the infected bacteria were diluted about 10 times in the radioactive medium (containing 1% Difco Neopeptone, 0.5% NaCl, 0.003% gelatin, 2% glycerol, and P^{32} in the form of orthophosphate in dilute HCl solution to obtain an activity of about 1 mc/mgP). The pH of the "hot" medium was adjusted to a value of 7 by the addition of NaOH. After 5 hr of growth and lysis, the stocks were freed of most of the contaminating P^{32} by three successive centrifugations at low and high speeds and resuspended in a protective medium containing glycerol and gelatin. The stocks made in this fashion contained only about one P^{32} atom per phage. The P^{32} stocks were used within 48 hr after their preparation. Control experiments utilizing the blendor technique of Hershey and Chase[7] (1952) have shown that most of the P^{32} adsorbed onto fresh bacteria is injected into them.

Gradient centrifugation: The method of gradient centrifugation and the assay of the phage distribution in the centrifuge tube by drop collection has been described elsewhere.[6] The Spinco preparative centrifuge was used with its swinging bucket rotor (SW39) and run at 22,000 rpm. The drops were collected in separate tubes containing 0.2 ml of Tryptone broth. An aliquot of 0.1 ml from each tube was put on planchettes for the measurements of the P^{32} content. Then convenient dilutions of the remaining volume were made, to determine the phage concentration in each tube. When crosses involved the markers *b*5 and $b5^+$, platings were made on bacteria carrying λ*b*5 and $\lambda b5^+$, respectively, and the numbers obtained were corrected for the plating efficiency relative to C600. (The efficiency of plating was usually about 0.80 for λ*b*5 on 3,110 lysogenic for $\lambda b5^+$.)

Genetic crosses: The crosses described were made in bacteria W3110. Cultures grown to a titer of 2×10^9 bacteria/ml were centrifuged and the bacterial pellets resuspended in adsorption medium and aerated at 37° for 1 hr. After this treatment, the bacteria were infected with an approximately equal number of both parental types of phages, the total multiplicity varying between 5.5 and 6.5 phages per bacterium in the different experiments. Ten minutes at 37° were allowed for adsorption (more than 95% adsorption) and the nonadsorbed phages as well as the small amount of free P^{32} still contained in the phage stocks were removed by centrifugation. The pellet of infected bacteria was resuspended at a titer of 2 to 4×10^8 cells/ml in fresh culture medium (some crosses were made in tryptone broth and some in *M* 9 AA), and the suspension was aerated at 37°. Time for lysis was between 70 and 150 min in different experiments. The lysates were centrifuged at low speed to eliminate bacterial debris, then at high speed to concentrate the phages and eliminate the free P^{32} in the supernatant. The phage pellets were resuspended in fresh medium and centrifuged a second time at high speed. Finally they were resuspended in a mall volume of Tris buffer at pH 7.6 before being diluted in CsCl for the density gradient centrifugation.

Results.—Crosses were made with the markers *b*2 and *b*5 in both coupling and repulsion. In each cross, one of the parents was labeled with P^{32}; experiments involving all of the four possible combinations of genotypes and P^{32} label have been carried out.

1. *Controls:* To show that during the growth of the phage of a single genetic

density type no other density mutants arise in amounts which would complicate the results of the crosses and that the presence of P^{32} does not affect the density distribution, the crosses $b2b5cP^{32} \times b2b5c$ and $b2^+b5^+cP^{32} \times b2^+b5^+c$ were first performed. The essential data concerning these are found in Table 1.

TABLE 1

Parents I	Parents II	Multiplicities of infection I	Multiplicities of infection II	Yield	Medium	Total transfer
$b2b5cP^{32}$	$\times b2b5c$	3.0	2.9	120	*M* 9 AA	0.43
$b2^+b5^+cP^{32}$	$\times b2^+b5^+c$	3.4	3.4	43	Tryptone	0.22

The density analyses by gradient centrifugation are shown in Figures 2 and 3. One sees that, essentially, no new densities appear in these crosses.‡ As has been pointed out,[1] the shape and width of the band obtained by collecting drops cannot be trusted if the band has been formed at or after drop number 40. Thus, although in general the bands of phages of lower densities seem to be wider than those of higher densities, no significance is to be attached to this fact.

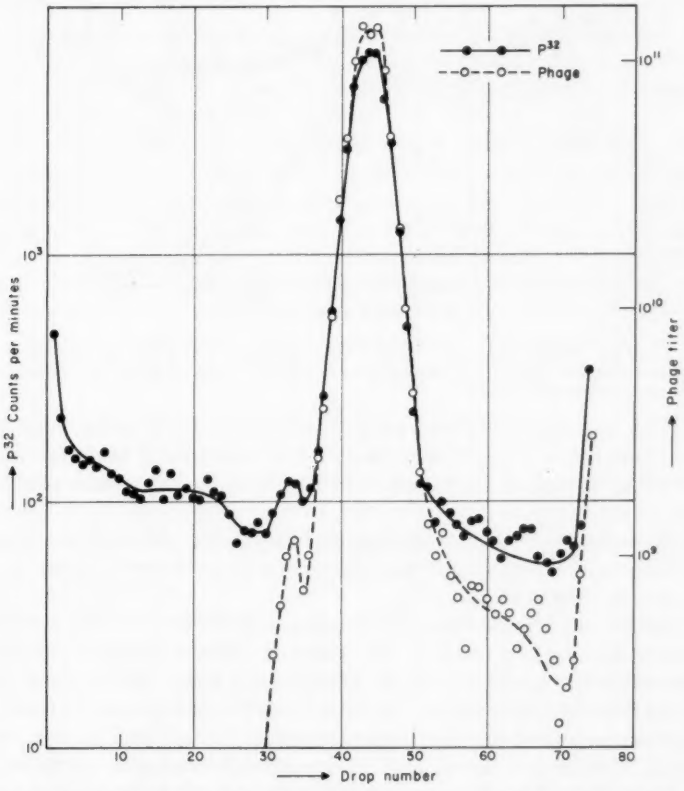

FIG. 2.—Control cross $\lambda b2b5cP^{32} \times \lambda b2b5c$. The two curves give, respectively, the titer of the progeny phage and the P^{32} contained in the drops collected after centrifugation.

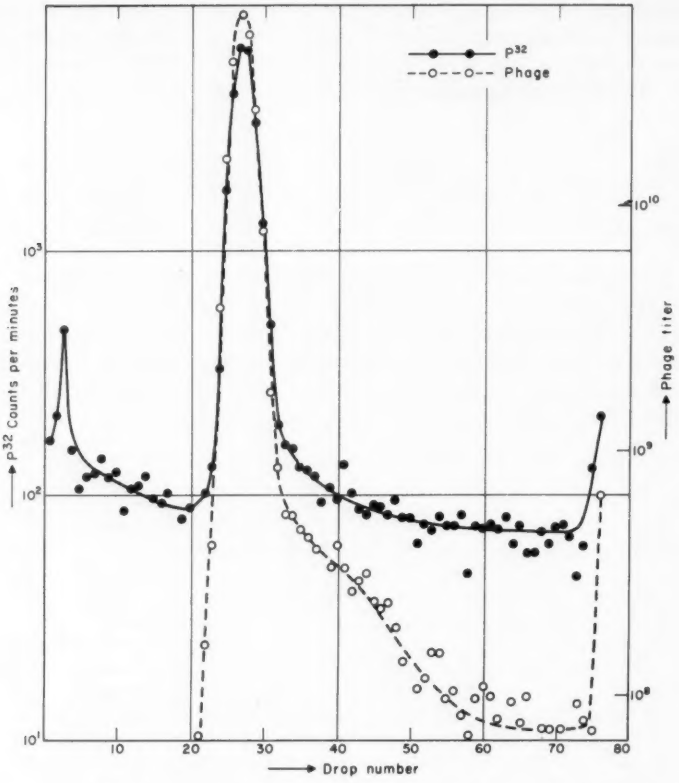

FIG. 3.—Control cross $\lambda b2^{+}b5^{+}cP^{32} \times \lambda b2^{+}b5^{+}c$. The two curves give, respectively, the titer of the progeny phage and the P^{32} contained in the drops collected after centrifugation.

The P^{32} is concentrated in the phage bands except for a background of radioactivity which appears in all the crosses. This background could be due to unfinished phage particles§ which burst and liberate their DNA when placed in the CsCl solution.

One concludes from the data of Figures 2 and 3 that the multiplication of the phages *b2b5* or $b2^{+}b5^{+}$ does not produce phages of a density different from that determined by their genotype.

2. *Transfer of P^{32}:* The amount of transfer of P^{32} from infecting particles to the progeny depends very much on the medium in which the crosses are performed. For instance in *M* 9 AA medium, lysis is delayed and a large part of the P^{32} remains in unlysed bacteria which can be centrifuged down at low speeds. At least 30 per cent and as much as 60 per cent of the introduced P^{32} can thus be lost. In these conditions, 30 to 50 per cent of the P^{32} is transferred to progeny particles adsorbable to bacteria and the remaining part of the P^{32} is found in the supernatant, probably in the form of free DNA or unfinished phages.

In tryptone broth, lysis is fairly complete but the phage yield is low and the

transfer is only about 20 per cent. As much as 75 per cent of the P^{32} can be found in the supernatant.

These data, based on many experiments, apply only when the lysing culture contains more than 2×10^8 bacteria/ml.

3. *Exchange of P^{32} in crosses of phages of different densities:* The data concerning the crosses $b2b5cP^{32} \times b2^+b5^+c$ and $b2^+b5^+cP^{32} \times b2b5c$ are found in Table 2 and the analysis of the progeny in the density gradient are represented in Figures 4 and 5. One sees in the figures that phages of two new bands of intermediate densities appear; they are the recombinants $b2^+b5$ and $b2b5^+$.

TABLE 2

Parents I	Parents II	Multiplicities of infection I	Multiplicities of infection II	Yield	Medium	Total transfer
$b2b5cP^{32}$	$\times b2^+b5^+c$	3.2	3.0	90	*M* 9 AA	0.52
$b2^+b5^+cP^{32}$	$\times b2b5c$	3.2	2.8	46	Tryptone	0.21

We have made use of the difference in immunity between $b5$ and $b5^+$ to obtain a better separation of the phage bands. The phages of each drop were plated on two indicator strains, one lysogenic for $\lambda b5$ and the other for $\lambda b5^+$: the plaques formed on the first strain give the count of $\lambda b5^+$ only while the second strain gives $\lambda b5$ only. Both curves were corrected for the plating efficiency with respect to the common indicator strain, UV-irradiated C600.

It will be noted in Figures 4 and 5 that the two recombinants do not appear in equal frequency. The $b2b5^+$ phage is two to three times as frequent as the reciprocal recombinant $b2^+b5$. We interpret this as meaning that $cb2b5^+$ has a selective advantage over the other phages involved in the crosses. When other markers such as *m*6, *mi*, etc. are associated with *c*, these phages do not have a selective advantage and behave in a "normal" fashion. Obviously, the advantage of $cb2b5^+$ gives an erroneous value of 20 per cent for the recombination frequency between $b2$ and $b5$. This same frequency is 10 per cent when the phages carry the markers mentioned above, the presence of which eliminates the selective advantage of $cb2b5^+$.

All the crosses mentioned in this paper have also been performed without the P^{32} label. The absence of P^{32} does not alter the recombination frequencies.

To make sure that no phenotypic mixing with respect to density had taken place in the crosses, the phages from the central drop of each peak were passed through one cycle of multiplication at low multiplicity of infection on bacteria W3110. The progeny was then centrifuged in the gradient along with additional phage to provide appropriate density markers. None of the four lysates obtained in this way contained more than 1 per cent of phages of a density different from that of the band from which they had been chosen.

P^{32} is found in all four types of progeny phages, i.e., in the two parents and in the two recombinants, but its distribution among the different types is very unequal. The largest part of the P^{32} remains with the parental genotype which brought it into the cross. The two recombinants contain approximately equal quantities and the progeny of cold parental genotype receives relatively little P^{32}. Although there are differences in the details of the distribution of P^{32} among the progeny depending on the parent which carried the P^{32} (see Fig. 6 for the other crosses $b2b5^+P^{32} \times$

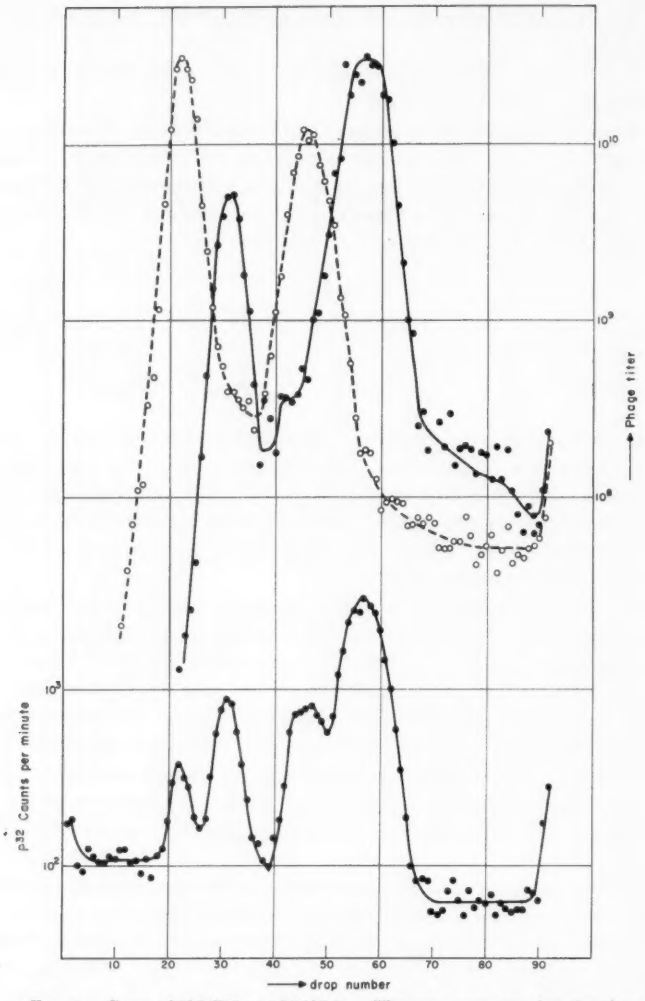

FIG. 4.—Cross $\lambda b2b5cP^{32} \times \lambda b2^+b5^+c$. The upper curves give the titer of the progeny phage in the drops collected after centrifugation. The peaks starting from the left represent the phage of the following genotypes $b2^+b5^+$, $b2^+b5$, $b2b5^+$, and $b2b5$. Since $\lambda b5$ and $\lambda b5^+$ have different immunities, selective indicator strains carrying the prophages $\lambda b5$ and $\lambda b5^+$ can be used to separate these two genotypes: on the indicator carrying $\lambda b5$, one obtains the measurements given by the interrupted line; on the other indicator, the continuous line. The lower curves give the P^{32} (in counts per minute) contained in the drops.

$b2^+b5$ and $b2b5^+ \times b2^+b5P^{32}$) the general distribution of P^{32} is always that described above.

To measure the total amount of P^{32} in each band, an approximate extrapolation was made for each peak and the area under each band measured (subtracting the background activity). These measurements are given in Table 3.

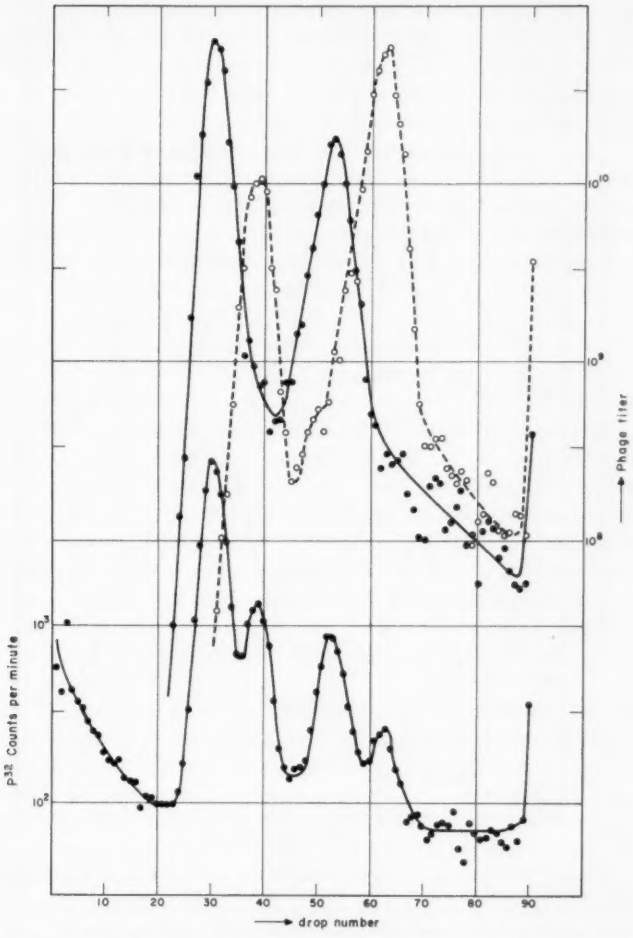

FIG. 5.—Cross $\lambda b2^{+}b5^{+}cP^{32} \times \lambda b2b5c$. The upper curves give the titer of the progeny phage contained in the drops collected after centrifugation. The lower curves give the P^{32} contained in the drops. For the details see the legend of Fig. 4.

TABLE 3

Cross	Radioactivity* in the Different Bands of Phage			
	$b2^{+}b5^{+}$	$b2^{+}b5$	$b2b5^{+}$	$b2b5$
I. $b2b5cP^{32} \times b2^{+}b5^{+}c$	1,171	4,071	4,308	26,035
II. $b2^{+}b5^{+}cP^{32} \times b2b5c$	35,100	5,285	4,245	998
III. $b2b5^{+}cP^{32} \times b2^{+}b5c^{+}$	1,303	303	8,311	1,194
IV. $b2^{+}b5c^{+}P^{32} \times b2b5^{+}cmi$	6,810	52,540	(2,524)	4,753

* As described in the text, the radioactivity, in counts per minute, was measured in each band of phages by adding all the counts under the band, the shape of the band being extrapolated from the curves giving the number of phages in each band. The background of radioactivity was subtracted as well as the background counts of the counter.

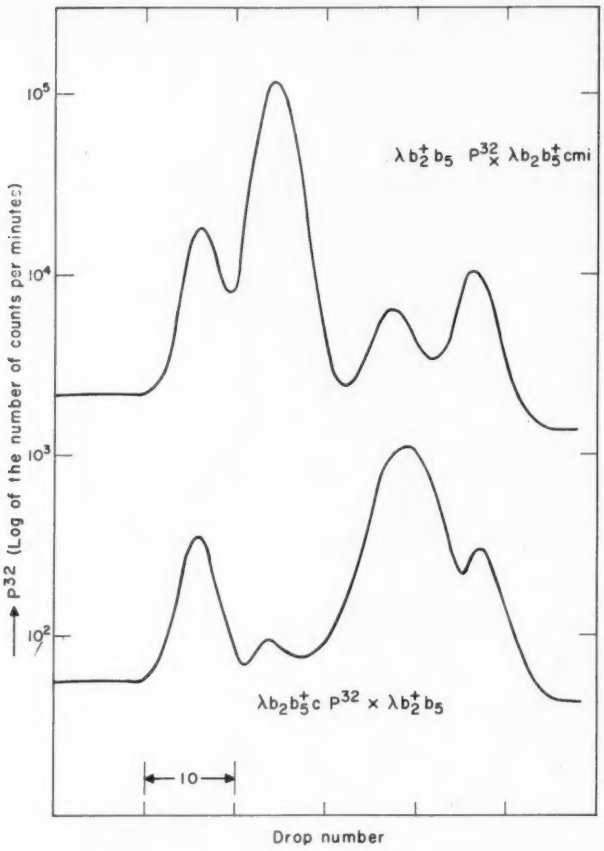

FIG. 6.—P^{32} contained in the drops, after centrifugation, of the crosses $\lambda b2^+b5P^{32} \times \lambda b2b5^+cmi$ (upper curve) and $\lambda b2b5^+P^{32} \times \lambda b2^+b5$. The positions of the peaks of the two curves do not quite coincide because the total number of drops collected in the two experiments were not equal. The logarithm of the number of counts per minute in the different drops is plotted as a function of the drop number. The upper curve has been arbitrarily displaced vertically.

In spite of the approximations involved, we estimate these measurements to be correct within 4 per cent except for the band $b2b5^+$ of the last cross of Table 3. For this cross, the "cold" parent band $b2b5^+$ is in the tail of the "hot" parent and a large correction must be applied. The error, in this case, may be as much as 50 per cent.

Discussion.—The experiments described above were designed to answer the following question: Are recombinant phage chromosomes made entirely of newly synthesized material or do they contain some of the original parental DNA material? A definite answer is given by our experiments: the recombinants *do* contain a large amount of the material from at least one of the infecting parents. Thus a second question can be asked: is the transferred material due to breakdown of

parental DNA and reutilization by the newly synthesized phage? If such were the process of transfer, one would expect to find at least as much P^{32} in the progeny with the genotype of the cold parental phage as in the recombinants. This is clearly not the case. The total amount of P^{32} in the cold parental progeny is only about 12 per cent of the total amount of P^{32} in the two recombinants; thus the parental DNA is transferred preferentially to the recombinants.

The simplest model accounting for these results is that recombination in λ occurs by breaks of the recombining chromosomes. This model allows one to calculate the relative amount of P^{32} brought to the progeny phages having the genotype of the cold parent by crossovers *outside* of the markers *b*2 and *b*5. Assuming that the amount of P^{32} in a chromosome is proportional to the genetic length and that there is only one break and knowing the position of *b*2 and *b*5 (see Fig. 1), one finds that the two recombinant genotypes (crossovers *between* *b*2 and *b*5) should contain 8 times more P^{32} than the progeny of the cold parental genotype. The data of the first three crosses (the data of the fourth cross cannot be used for the reason given above) given for the ratio of the total amount of P^{32} in the recombinant to that contained in the cold parental genotype the value 8 ± 1.

The good agreement between the estimated and the observed ratio leads us to think that most, if not all, of the P^{32} is transferred by crossovers and not by breakdown and reutilization. This estimate is based on the fact that crossovers are rare in λ and that double crossovers can be neglected. It may be that further studies of the mechanism of breaks will need a more detailed analysis of our data.

All these considerations are based on the total amount of P^{32} found in the recombinants and the cold parental genotypes, respectively. They are thus *independent of the mating theory.*

The nature of the density markers *b*2 and *b*5 has so far been left unspecified. These markers simply gave a definite phenotype to the phages harboring them, and in a cross they behaved like any other genetic marker. If one takes into account the fact that $b2^+$ and probably $b5^+$ contain more DNA than *b*2 and *b*5, the data of Table 3 contains the information necessary to calculate, under certain assumptions, the amounts of the supplementary DNA. If one assumes (1) that the extra DNA of each marker is situated at the location of the marker itself, and (2) that the presence of the extra DNA does not significantly alter the genetic exchanges between the homologous sections of the two genomes, the different relative amounts of P^{32} brought into the recombinant by a $P^{32}b2^+b5^+$ and by a $P^{32}b2b5$ phage lead to the following results: λ $b2^+b5$ contains 17 per cent, and λ $b2b5^+$ contains 6 per cent more DNA than λ *b*2*b*5. The assumption that the density difference between these phages is due entirely to their DNA content would have given, according to the formula (1) of Weigle *et al.*,[6] the values 17 per cent and 7 per cent, respectively.

Conclusions.—In genetic crosses between phage λ of different densities, the recombinants can be physically separated from the parents. When one of the parents is labeled with P^{32} and the genetic markers are far apart, most of the P^{32} transferred to the progeny of genotypes different from that of the hot parent is found in the recombinants. Breakdown and reutilization of the parental material is probably negligible.

The simplest model accounting for these facts is one in which the recombinants are formed by breaks of the two parental materials.

* Aided by a grant from the Swiss National Foundation for Scientific Research.
† Aided by a grant from the National Foundation.
‡ The small band appearing on the dense side of *b2b5* in Fig. 2 contains only 0.2% of the phages; it is not due to an error in collecting the drops, for if the phages are centrifuged again in the density gradient, these phages appear at the same density; however, after one cycle of multiplication they give progeny phages of the density *b2b5*. The reason for this behavior is unknown.
§ Observed in λ lysates by E. Kellenberger in the electron microscope.

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ON THE CLASS NUMBER OF REAL QUADRATIC FIELDS

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THEOREM.—*Let p be a prime $\equiv 1 \pmod 4$, h the class number of the real quadratic field $R(\sqrt{p})$, $\epsilon = [(t + u\sqrt{p})/2] > 1$ its fundamental unit. Then,*

$$\left(\frac{p-1}{2}\right)! \equiv (-1)^{(h+1)/2}\frac{t}{2} \pmod p. \tag{1}$$

Proof: From Dirichlet's classical formula we derive

$$\sqrt{p}\,\epsilon^h = \prod_n (1 - \theta^n), \tag{2}$$

where $\theta = e^{2\pi i/p}$ and n runs over the numbers with $0 < n < p$ and $(n/p) = -1$, where (x/p) is Legendre's symbol of quadratic residuacity. Working with integers of $R(\theta)$, we note

$$\sqrt{p} = \left(\frac{p-1}{2}\right)!\,(1-\theta)^{(p-1)/2} \pmod{(1-\theta)^{(p+1)/2}}, \tag{3}$$

$$\prod_n (1 - \theta^n) = (1-\theta)^{(p-1)/2} \pmod{(1-\theta)^{(p+1)/2}}. \tag{4}$$

(1) follows from (2), (3), and (4). Our result supplements formulae of Ankeny-Artin-Chowla in *Ann. Math.*, 1952, p. 479.

I am grateful to Professor A. Selberg for noticing an error in my original argument. Paromita Chowla has checked the formula in many special cases. See L. J. Mordell, *Amer. Math. Monthly* (Feb. 1961) for a similar result for primes $p \equiv 3 \pmod 4$.

*ASYMPTOTIC BEHAVIOR AND PERIODIC SOLUTIONS OF A NONLINEAR DIFFERENTIAL-DIFFERENCE EQUATION**

By G. Stephen Jones

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Communicated by S. Lefschetz, March 29, 1961

1. *Introduction.*—This paper concerns the nonlinear differential-difference equation

$$f'(x) = -\alpha f(x-1)\{1+f(x)\}, \qquad x>0 \tag{1.1}$$

where α is a real positive parameter. If we let ϕ be a real-valued, bounded, and Lebesgue-integrable function defined on $(-1, 0]$, then a function f such that $f(x) = \phi(x)$ for x in $(-1, 0]$ and

$$f(x) = f(0) - \alpha \int_0^x f(t-1)\{1+f(t)\}dt,$$

for $x > 0$ is referred to as a solution of (1.1) corresponding to the initial function ϕ.

Equation (1.1) and closely related ones occur widely in application. For example, it occurs in the application of probability methods to the distribution of prime numbers[1] and in a mathematical description of a fluctuating population of organisms.[2] It is also encountered in describing control systems, and quite similar equations arise in economic studies of business cycles.[3]

Wright[4] has demonstrated the existence and uniqueness of a solution of (1.1) corresponding to each initial function, and, in fact, has presented the formula

$$f(x) = \{1+f(0)\}\exp\left\{-\alpha\int_{-1}^{x-1} f(t)dt\right\} - 1, \tag{1.2}$$

from which f can be successively computed on unit intervals. He has also proved the asymptotic stability of the trivial solution for $0 \leqq \alpha \leqq {}^3/_2$, the existence of undamped bounded oscillatory solutions for $\alpha > (\pi/2)$, and a large number of other interesting results including several expansions for solutions. Kakutani and Markus[5] have presented theorems separating the strict asymptotic behavior of solutions for α in $(0, e^{-1})$ and the oscillatory behavior which occurs for $\alpha > e^{-1}$.

The further development of the theory of (1.1) indicated in this paper is concerned with the undamped oscillatory solutions which occur for $\alpha > (\pi/2)$. Improved bounds on amplitudes and restrictions on the behavior of solutions are stated. The larger portion, however, is directed toward establishing the existence of nonconstant periodic solutions and some of their properties. Some numerical results, obtained with the aid of a computer, strongly suggesting that solutions for $\alpha > (\pi/2)$ rapidly approach a fixed periodic form, are mentioned.

2. *The Existence and Properties of Periodic Solutions.*—We define $C[-1, 0]$ to be the metric space consisting of the set of all continuous functions defined on $[-1, 0]$ together with the metric $\rho(\phi_1, \phi_2) = \max\{|\phi_1(x) - \phi_2(x)|\}$. $S \subset C[-1, 0]$ is defined to be the set of continuously differentiable functions such that ϕ in S implies $\phi(-1) = 0$, $\phi(x) \not\equiv 0$, $0 \leqq \phi(x) \leqq e^{\alpha} - 1$, and $0 \leqq \phi'(x) \leqq \alpha e^{\alpha}$. Unless otherwise indicated, f shall henceforth denote a solution of (1.1) corresponding to an initial function in S. From the results of Wright, we have the following properties:

(2.1) For $\alpha > 0$ and $x > 0$, $-1 < f(x) < e^{\alpha} - 1$.

(2.2) For $\alpha > e^{-1}$, the set of zeros of f is a countably infinite set $z_1 < z_2 < \ldots$, and $z_{n+1} - z_n > 1$ for all n.

(2.3) For $\alpha > e^{-1}$, the sequence $\{z_n + 1\}$ is the complete set of zeros of f'.

(2.4) If $\alpha > 1$ and $|f(z_n + 1)|$ is sufficiently small, then $z_{n+1} - z_n < 3$.

(2.5) For $\alpha > (\pi/2)$, f oscillates about θ (the trivial solution) but does not tend to zero as $x \to \infty$.

We now present a sequence of simply established lemmas which are instrumental in establishing the principal theorems.

2.1. LEMMA. Let $\alpha > 0$ and $f(0) > -1$. Let x_1 and x_2 be points such that $x_2 > x_1 > 0$. Then,

$$f(x_1) \gtreqless f(x_2) \quad \text{implies} \quad \int_{x_1-1}^{x_2-1} f(t)dt \gtreqless 0,$$

and

$$f(x_1) = f(x_2) \quad \text{implies} \quad \int_{x_1}^{x_2} f(t)f(t-1)dt = 0.$$

2.2. LEMMA. If $\alpha > e^{-1}$ and $\lambda_n = z_{n+1} - z_n$, then $\lambda_n = 1 + |M_n|/|M_n^*|$, where M_n and M_n^* are the mean values of f on $(z_n - 1, z_n)$ and $(z_n, z_{n+1} - 1)$ respectively.

2.3. LEMMA. Let $\lambda = \sup\{\lambda_n\}$ be considered a functional defined for ϕ in S. λ is bounded on S.

2.4. LEMMA. Let T: $\bar{S} \to C[-1, 0]$ be such that $T(\theta) = \theta$ and $T[\phi(x)] = f(\lambda_2 + 1 + x)$ for ϕ in S and x in $[-1, 0]$. T maps $\bar{S}$ into $\bar{S}$ topologically.

Since f is uniquely determined by its specification on a unit interval, it follows that if there exist ϕ in S and finite n such that the nth-power composite mapping of ϕ under T is ϕ, then the solution of (1.1) corresponding to ϕ is periodic.

Let $S(\epsilon) \subset S$, ϵ in $(0, e^{\alpha} - 1)$, be such that ϕ in $S(\epsilon)$ implies $\phi(0) \geqq \epsilon$. If we can show for a properly chosen ϵ and n that $T^n(S(\epsilon)) \subset S(\epsilon)$, then the existence of the desired fixed point follows from the Tychonoff fixed-point theorem.[6]

Let $S^*(\epsilon) \subset S$ be such that ϕ in $S^*(\epsilon)$ implies $\phi(0) = \epsilon$. The following theorem concerning the asymptotic behavior of a related linear differential-difference equation is fundamental.

2.5. THEOREM. For g a continuous function and $x > 0$, let $U(\epsilon)$ denote the set of solutions of the equation

$$u'(x) = -\alpha u(x - 1) + g(x)$$

corresponding to initial functions in $S^*(\epsilon)$, and let there exist positive constants β and δ such that

$$|g(x)| \leqq \beta\epsilon^{1+\delta} \quad \text{or} \quad \left|\int_0^x g(t)dt\right| \leqq \beta\epsilon^{1+\delta}.$$

Then for $\alpha > (\pi/2)$, and $\epsilon_1 > 0$ sufficiently small, solutions oscillate about θ, and there exists a constant x^* such that for arbitrary u in $\cup\{U(\epsilon) : \epsilon \text{ in } (0, \epsilon_1]\}$, $u(x)$ exceeds ϵ at some point in every interval contained in $\{x: x \geqq x^*\}$ over which $u(x)$ is positive.

With the aid of the extensive results concerning the equation $y'(x) = -\alpha y(x - 1)$ contained in Wright's paper, the proof of Theorem 2.5 is not difficult.

Now through a sequence of lemmas we indicate a means by which the inclusion $T^n(S(\epsilon)) \subset S(\epsilon)$ can be established for some finite n and $\epsilon > 0$. In this connection,

it is convenient to define the set $\hat{S}(\epsilon) = [S - S(\epsilon)] \cup S^*(\epsilon)$.

2.6. LEMMA. For $\alpha > 0$, let f correspond to ϕ in $\hat{S}(\epsilon_1)$, $1 > \epsilon_1 > 0$. Let $x(\phi) > 0$ be the first point where $\rho(f, \theta) \geqq \epsilon_1^{3/4}$. Then for an arbitrary positive constant K, there exists $\epsilon_1 > 0$ such that $x(\phi) > K$ for all ϕ in $\hat{S}(\epsilon_1)$.

2.7. LEMMA. Let ψ be an arbitrary element of $T^n(S(\epsilon))$ for any $n \geqq 1$. Then there exists ψ^* in $T^n(S^*(\epsilon))$ such that $\rho(\psi, \theta) \geqq \rho(\psi^*, \theta)$.

2.8. LEMMA. If $\alpha > (\pi/2)$, then for sufficiently small ϵ there exists n_1 such that $T^m(S(\epsilon)) \subset S(\epsilon)$ for all n such that $n_1 \leqq n \leqq m$ where m can be arbitrarily large.

Theorem 2.5 is the principal tool in establishing Lemma 2.8. Our existence theorem now follows easily and is formally stated as follows:

2.9. THEOREM. There exist nonconstant periodic solutions of (1.1) for all $\alpha > (\pi/2)$.

From results previously stated, it is easily shown that if f is a periodic solution of period λ, then for arbitrary real x, $\int_x^{x+\lambda} f(t)dt = 0$. Also, from Lemma 2.8 it follows that if $T(\phi) \neq \phi$ for all ϕ in S, then there exist an infinite number of distinct periodic solutions of (1.1) for each $\alpha > (\pi/2)$. From a result presented in Wright's paper we can deduce that every periodic solution is analytic in a strip of the complex plane containing the real line.

3. *Bounds and Restrictions on Asymptotic Behavior.*—A large number of interesting results can be obtained bounding and restricting the behavior of solutions of (1.1) various ways. Some of the more simply describable ones will now be stated.

3.1. THEOREM. Let $\alpha \geqq (\pi/2)$ and let $z_n > 2$ in $\{z_n\}$ be such that $f(x) > 0$ on (z_n, z_{n+1}). $f(x) > e^{\alpha(x-z_n)} - 1$ for x in $(z_n - 1, z_n)$, $f(x) < \exp\{\alpha(x - z_n) + e^{-\alpha} - e^{\alpha(x-z_n-1)}\} - 1$ for x in $(z_n, z_n + 1)$, and $f(x) < \exp\{\alpha + e^{-\alpha} - 1\} - 1$ for $x > \max\{1, z_{n-1}\}$. $z_{n+1} - z_n > 1 + \frac{1}{\alpha}$, $f(x) < \exp\{\exp\{\alpha + e^{-\alpha} - \exp\{\alpha(x - z_{n+1} - 1) + 1\}\} + \alpha(x - z_{n+1} + 1) + e^{-\alpha} - e^{\alpha}\} - 1$ for x in $[z_{n+1} - \frac{1}{\alpha}, z_{n+1}]$, and if $\tilde{M}$ denotes the mean value of $f(x)$ on $[z_{n+1} - 1, z_{n+1}]$, $\tilde{M} < \frac{1}{\alpha}\{(\alpha - 1)\exp\{\alpha + e^{-\alpha} - 1\} + \alpha \exp\{\alpha + e^{-\alpha} + 1 - e\}\} - 1$. $f(x) > \exp\{\alpha - (\alpha - 1)\exp\{\alpha + e^{-\alpha} - 1\} - \alpha \exp\{\alpha + e^{-\alpha} + 1 - e\}\} - 1$, for $x \geqq z_n$.

The function, $F(x) = \exp\{\alpha x + \exp\{\alpha + e^{-\alpha} - e^{-\alpha(x-2)}\} - \exp\{\alpha + e^{-\alpha} - e^{-2\alpha}\}\} - 1$ from $x = 0$ to the second positive zero of F, is of interest in that it closely approximates the numerically computed periodic solutions of (1.1) for corresponding α. This can be verified by comparing computed values of F with corresponding values taken from the curves exhibited in Figure 1.

3.2. THEOREM. Let $\alpha > (\pi/2)$ and let f correspond to an initial function in S. Then for each z_n in $\{z_n\}$ there exists a neighborhood N_n of z_n of length l_n and a positive constant ξ such that $\inf\{\ell_n\} > 0$ and $|f'(x)| > \xi$ for all x in $\cup\{N_n\}$.

3.3. THEOREM. Let $\alpha > e^{-1}$ and let z_n in $\{z_n\}$ be such that $n > 1$ and $f(x) > 0$ for x in (z_n, z_{n+1}). Then there exist x_1 in $(z_{n-1} + 2, z_n + 1)$ and x_2 in $(z_n + 1, z_n + 2)$ such that $f''(x_1) = f''(x_2) = 0$. Also $f''(x) \neq 0$ for x in $[z_n + 2, z_{n+1} + 2]$, and if $f''(x_1) = 0$ and $x_1 > 2$, then $f(x_1 - 2) < 0$.

4. *Numerical Indications.*—Using the formula $f(x) = [1 + f(x - 1)] \exp\{-\alpha \int_{x-2}^{x-1} f(t)dt\} - 1$ and numerical integration, a number of solutions of (1.1)

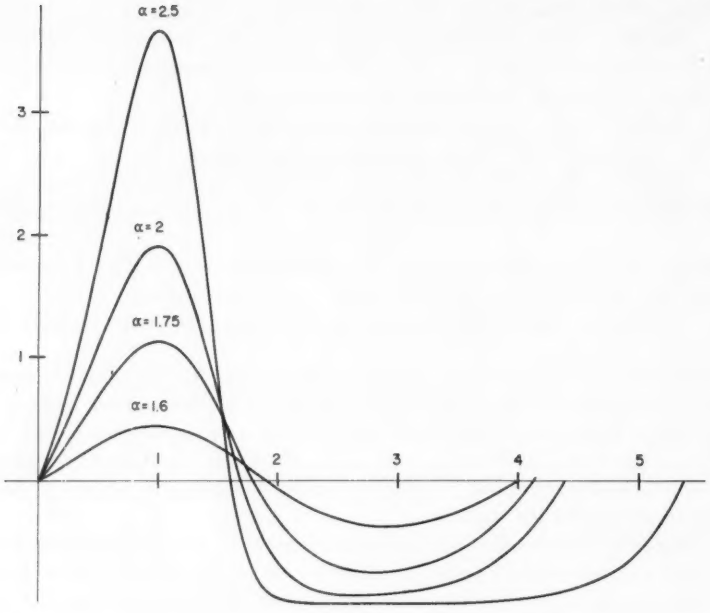

FIG. 1.

were computed for various values of α and a variety of initial functions. The results thus obtained strongly indicate that for $\alpha > (\pi/2)$ there exists ϕ^* in S such that $T(\phi^*) = \phi^*$, and all solutions of (1.1) corresponding to initial functions in S asymptotically approach the periodic form implied by this fixed point. ϕ^* appears to depend only on α, and the effect of choosing different initial functions in S is only a shift of phase. The computed periodic forms corresponding to several values of α are given in Figure 1.

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A METHOD FOR DEFINING PRINCIPAL MODES OF NONLINEAR SYSTEMS UTILIZING INFINITE DETERMINANTS, II

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1. (*a*) In a recent paper,[1] the writer has discussed the "principal modes" of a "dual-mode" nonlinear system utilizing "infinite determinants." However, this paper did not give the calculation of the infinite determinants involved and the solution in its final form; also, it did not give the "principal modes" defined as solutions of special "initial-value problems." This is the object of the present paper.

(*b*) We can state for reference that in a "dual-mode" nonlinear system, examined in the previous paper, the "principal modes" are given by

$$\chi(t) = \sum_{n=-\infty}^{\infty} \alpha_n e^{in\omega t}, \qquad \psi(t) = \sum_{n=-\infty}^{\infty} \frac{\lambda_3}{\omega_2^2 - n^2\omega^2}\, \alpha_n e^{in\omega t}, \tag{1}$$

and the "recursion formula," for the calculation of the "principal frequency" ω and the coefficients α_n, is

$$p_n\alpha_{n-2} + \alpha_n + q_n\alpha_{n+2} = 0 \tag{2}$$

where

$$p_n = \frac{3\lambda_1\alpha_1^2}{k_n + 6\lambda_1|\alpha_1|^2}, \quad q_n = \frac{3\lambda_1\bar{\alpha}_1^2}{k_n + 6\lambda_1|\alpha_1|^2}, \quad k_n = \omega_1^2 - n^2\omega^2 - \frac{\lambda_2\lambda_3}{\omega_2^2 - n^2\omega^2}. \tag{2a}$$

The factors 3, 6, 3 of the expressions (2*a*) are the coefficients of the trinomial (A_1) of the Appendix, which gives the dominant sum of the double series of the formula:

$$k_n\alpha_n + \lambda_1 \sum_{\rho_1} \sum_{\rho_2} \alpha_{\rho_1}\alpha_{\rho_2}\alpha_{n-\rho_1-\rho_2} = 0. \tag{3}$$

For the convergence of the series (1), it is necessary that $(n^2\omega^2 - \omega_2^2)$ be neither zero nor very small, i.e., ω must not be either a submultiple of ω_2 or very close to a submultiple of ω_2.

2. The "frequency equation" for the calculation of the "principal frequency" ω comes from the condition of nonzero α_n's of the infinite system (2). This condition in a determinatal form is

$$\Delta(n, \infty) = 0, \tag{4}$$

and the determinant $\Delta(n, \infty)$, for arbitrary integer n, is the following one-sided infinite determinant:

$$\Delta(n, \infty) = \lim_{\substack{n = \text{fixed integer} \\ m = 1, 2, 3, \ldots \\ m \to \infty}} \Delta(n, m) \equiv \begin{vmatrix} 1 & 0 & q_n & 0 & \cdots & 0 \\ 0 & 1 & 0 & q_{n+1} & 0 \cdots & 0 \\ p_{n+2} & 0 & 1 & 0 & q_{n+2}\ 0 \cdots & 0 \\ \cdots & \cdots & \cdots & \cdots & \cdots & \cdots \\ 0 \cdots & 0 & 1 & 0 & q_{n+m-2} & \\ 0 \cdots & p_{n+m-1} & 0 & 1 & 0 & \\ 0 \cdots & 0 & p_{n+m} & 0 & 1 & \end{vmatrix} \tag{5}$$

Consider a weak nonlinearity, i.e., $\lambda_1 |x|^3 \ll \omega_1^2 |x|$. Then, since α_1 is the dominant coefficient of the series (3), $\max x^2 = 2\alpha_1^2$ when

$$\lambda_1 \ll \frac{K_1 + K_2}{2m_1\alpha_1^2}. \tag{6a}$$

For such a small λ_1, we can get

$$p_n = \lambda_1 \frac{3\alpha_1^2}{k_n} - \lambda_1^2 \frac{18\alpha_1^2 |\alpha_1|^2}{k_n^2} + \ldots, \quad q_n = \lambda_1 \frac{3\bar{\alpha}_1^2}{k_n} - \lambda_1^2 \frac{18\bar{\alpha}_1^2 |\alpha_1|^2}{k_n^2} + \ldots \tag{6}$$

Thus, the elements of the determinant (5) not in the main diagonal have the small λ_1 as a common factor, and we can get an expansion of the determinant in powers of λ_1. Following the appropriate way to get this expansion,[2] by taking the first terms of (6), we obtain

$$\Delta(n, \infty) = 1 - 9\lambda_1^2 |\alpha_1|^4 \sum_{m=0}^{\infty} \frac{1}{k_{n+m} \cdot k_{n+m+2}} + 0(\lambda_1^3) \tag{7}$$

For any ω, which is not a zero of the denominators of the elements of the series in (7), this series converges. Combining (4) and (7), if we confine ourselves to the first term of the series in (7), and putting $n = 1$, the frequency equation becomes approximately the following equation, quartic in ω^2:

$$\left(\omega_1^2 - \omega^2 - \frac{\lambda_2\lambda_3}{\omega_2^2 - \omega^2}\right)\left(\omega_1^2 - 9\omega^2 - \frac{\lambda_2\lambda_3}{\omega_2^2 - 9\omega^2}\right) - 9\lambda_1^2 |\alpha_1|^4 = 0, \tag{8}$$

which gives the principal frequency ω of order 0 (λ_1^2). For values of the principal frequency of order higher than 0 (λ_1^2), we find in the expansion of $\Delta(n, \infty)$ terms of order higher than 0 (λ_1^2) and continue in the same way to find the corresponding algebraic equation.

3. The calculation of the coefficients α_n is based on the following formula of the previous paper:[1]

$$\alpha_n/\alpha_{n-2} = -p_n \cdot \cfrac{1}{1 + \cfrac{X_{n,1}}{1 + \cfrac{X_{n,2}}{1 + \ldots}}} \tag{9}$$

with

$$X_{n,m} = -p_{n+2m} \cdot q_{n+2(m-1)}$$

$$n \quad = \text{any fixed integer, } m = 1,2,3, \ldots \tag{9a}$$

By using the product of m linear transformations of the form

$$X_1(v) = 1, \quad X_m = \frac{1}{1 + X_m v}, \; m = 2,3,4, \ldots$$

$$X_p \neq 0, \; p = 2, 3, \ldots m; \quad X_p = 0, \; p > m, \tag{10}$$

one can prove that the m^{th} approximant of the continued fraction of (9) may be equal to the ratio of two determinants of which the denominator is

$$\bar{\Delta}(n,m) =$$

$$\begin{Vmatrix} 1 & -1 & 0\,. & 0 & 0 & 0 \\ -p_{n+2}q_n & 1 & -1\,. & 0 & 0 & 0 \\ 0 & -p_{n+4}\cdot q_{n+2} & 1\,. & 0 & 0 & 0 \\ \cdots & \cdots & \cdots & \cdots & \cdots & \cdots \\ 0 & 0 & 0\,. & -p_{n+2(m-1)}\cdot q_{n+2(m-2)} & 1 & -1 \\ 0 & 0 & 0\,. & 0 & -p_{n+2m}\cdot q_{n+2(m-1)} & 1 \end{Vmatrix} \tag{11}$$

and the numerator, $\bar{\Delta}(n+2,m)$, is obtained from (11) by omitting its first row and first column. For fixed n, and $m \to \infty$, we can write

$$\alpha_n/\alpha_{n-2} = -p_n \frac{\bar{\Delta}(n+2,\infty)}{\bar{\Delta}(n,\infty)} \tag{12}$$

where the infinite determinants are of von Koch's type.

The induction procedure applied to (12) gives for the coefficients with even index:

$$\alpha_{2n} = (-1)^n \alpha_0 p_2 p_4 \ldots p_{2n} \cdot \frac{\bar{\Delta}(2n+2,\infty)}{\bar{\Delta}(2,\infty)}, \; n = 1,2,3, \ldots \tag{13a}$$

For coefficients with odd index, starting from

$$\alpha_3 = -\lambda_1 \frac{\alpha_1^3}{k_3}, \tag{13b}$$

which can be found by using (3) and the Appendix, we have

$$\alpha_{2n+1} = (-1)^{n+1}\lambda_1 \frac{\alpha_1^3}{k_3} \cdot p_5 p_7 \ldots p_{2n+1} \frac{\bar{\Delta}(2n+3,\infty)}{\bar{\Delta}(5,\infty)}, \quad n = 2,3, \ldots, \tag{13c}$$

α_0 and α_1 are arbitrary coefficients. By using the property $\alpha_{-n} = \bar{\alpha}_n$ and taking α_1 real, the solution (1) can be written as follows:

$$\chi(t) = \alpha_0 + 2\alpha_1 \cos \omega t - 2\alpha_3 \cos 3\omega t + 2 \sum_n \alpha_N \cos N\omega t,$$

$$\psi(t) = \frac{\lambda_3}{\omega_2^2}\alpha_0 + \frac{2\lambda_3\alpha_1}{\omega_2^2 - \omega^2}\cos \omega t - \frac{2\lambda_3\alpha_3}{\omega_2^2 - 2^2\omega^2}\cos 3\omega t + 2\sum_n \frac{\lambda_3\alpha_n}{\omega_2^2 - N^2\omega^2}\cos N\omega t, \tag{14}$$

where α_3, $\alpha_{N=2n}$, and $\alpha_{N=n+1}$ are given by (13b), (13a), and (13c), respectively. Formulas (14) give the solution in its final form, and by using (13a), (13b), and (13c), we can calculate as many coefficients of the solution (14) as we want, in terms of powers of λ_1. By expanding the determinants of (13a) and (13c) in powers of the small λ_1,[2] we find that their ratios are equal to $1 + 0\,(\lambda_1^2)$, and, as we can easily see, the calculation of the coefficients, in terms of powers of λ_1, needs only the unit as value of these ratios. The first two terms of (14) are independent of λ_1. For terms of (14) of order $O\,(\lambda_1)$, we take the first term of p_n of (6) and, combining it with (13a) for $n = 1$, we get the 2nd harmonic terms of the series (14), when the terms of order $O\,(\lambda_1)$ are

$$-2\lambda_1\left\{\frac{3\alpha_0\alpha_1^2}{k_2}\cos 2\omega t + \frac{\alpha_1^3}{k_3}\cos 3\omega t\right\}, \text{ of } \chi(t).$$

$$-2\lambda_1\left\{\frac{3\lambda_3\alpha_0\alpha_1^2}{k_2(\omega_2^2 - 2^2\omega^2)}\cos 2\omega t + \frac{\lambda_3\alpha_1^3}{k_3(\omega_2^2 - 3^2\omega^2)}\cos 3\omega t\right\}, \text{ of } \psi(t). \qquad (14a)$$

If we take the terms of p_n of (6) up to the order $O(\lambda_1^2)$ and combine them with (13a) for $n = 2$, we obtain the 4th harmonic terms of order $O(\lambda_1^2)$:

$$18\lambda_1^2\frac{\alpha_0\alpha_1^4}{k_2k_4}\cos 4\omega t, \quad 18\lambda_1^2\frac{\alpha_0\alpha_1^4\lambda_3}{k_2k_4(\omega_2^2 - 4^2\omega^2)}\cos 4\omega t \qquad (14b)$$

of $\chi(t)$ and $\psi(t)$, respectively. Following the same way, we can calculate the higher harmonics in terms of higher powers of λ_1. The solution (14), constructed as indicated, must be convergent, and its coefficient of the fundamental term must be much larger than any other coefficient. These requirements imply that the following conditions are satisfied:

$$\alpha_0 << \alpha_1, \quad \lambda_1 << \min\left\{\frac{K_1 + K_2}{2m_1\alpha_1^2}, \frac{k_2}{2\alpha_0\alpha_1}, \frac{k_3}{\alpha_1^2}\right\}. \qquad (15)$$

For the second condition, the inequality (6a) was taken into account. The 4th, 5th, . . . harmonic terms are of order $O(\lambda_1^2)$, $O(\lambda_1^3)$, . . . , and the convergence is guaranteed.

4. Another approach for the determination of principal modes is based on the manner in which the system is set into motion. This is equivalent to considering the principal modes as solutions of special initial-value problems. The solution (14), found in the previous section, gives an indication about the appropriate initial conditions. The initial displacements are, according to (14), the sum of the coefficients, and the initial velocities are zero.

Consider the differential equations of the "dual-mode" nonlinear system subject to the two sets of initial conditions:

$$\chi(0) = \chi_0, \quad \psi(0) = \psi_0, \quad \dot{\chi}(0) = \dot{\psi}(0) = 0 \qquad (16a)$$

$$\chi(0) = \chi_0, \quad \psi(0) = -\psi_0, \quad \dot{\chi}(0) = \dot{\psi}(0) = 0. \qquad (16b)$$

If the initial displacements χ_0 and ψ_0 are appropriately related, the initial velocities being zero, then each set of the initial conditions (16a) or (16b) gives rise to special vibration modes, which are, by definition, the principal modes of the system. If we assume the series

$$\chi(t) = \sum_{n=-\infty}^{\infty}\alpha_n e^{in\omega t}, \quad \psi(t) = \sum_{n=-\infty}^{\infty}A_n e^{in\omega t} \qquad (17)$$

as solution of the differential equations of the system, by repeating the previous procedure, we will terminate to get the solution in the form (14), which gives the appropriate relationship between the initial displacements of the two oscillators. An approximation of this solution for very small λ_1, and α_1 as dominant coefficient, is:

$$\chi(t) = 2\alpha_1\cos\omega t, \quad \psi(t) = 2\frac{\lambda_3}{\omega_2^2 - \omega^2}\alpha_1\cos\omega t, \qquad (18)$$

where the initial conditions are

$$\chi_0 = 2\alpha_1, \quad \psi_0 = \frac{\lambda_3}{\omega_2^2 - \omega^2}\chi_0, \quad \dot{\chi}_0 = \dot{\psi}_0 = 0. \tag{18a}$$

Formula (18*a*) give the initial conditions required for the solution to be of "principal modes" type.

Appendix.—If in the elements of the sequence $\{\alpha_n\}$. $n = 0, \pm 1, \pm 2, \ldots$, where $\alpha_{-n} = \bar{\alpha}_n$, the element α_1, and then α_{-1}, is dominant, it is easily seen that the dominant sum of the double series of (3) is given by

$$3\alpha_1^2\alpha_{n-2} + 6|\alpha_1|^2\alpha_n + 3\bar{\alpha}_1^2\alpha_{n+2}, \tag{A_1}$$

where n is any integer except $n = \pm 1, \pm 3$. For these exceptions, the dominant terms of the double series are

$$3\bar{\alpha}_1\alpha_1^2 \quad \text{for } n = 1, \qquad 3\alpha_1\bar{\alpha}_1^2 \quad \text{for } n = -1, \qquad \alpha_1^3 \text{ for } n = 3, \bar{\alpha}_1^3 \quad \text{for } n = -3. \tag{A_2}$$

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RESPIRATORY DISEASE IN VOLUNTEERS INFECTED WITH EATON AGENT; A PRELIMINARY REPORT

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Recent epidemiologic studies have provided evidence that the filterable agent first described by Eaton in 1944 was associated with human respiratory disease[1-3] A controlled field study among Marine recruits suggested that the Eaton agent caused a spectrum of disease including febrile respiratory illness and atypical pneumonia.[3] In the same Marine recruit population a double blind controlled study indicated that demethylchlortetracycline was effective in the therapy of pneumonia associated with Eaton infection.[4] During the course of these studies 14 strains of the agent were recovered in monkey kidney tissue culture.[5]

As a further step in assessing the etiologic role of Eaton agent in respiratory disease, tissue culture grown material was administered to volunteers in an attempt to reproduce the natural disease. This approach was made possible by the availability of an effective therapeutic drug and freshly isolated strains of the agent. This communication will present a preliminary analysis of the Eaton volunteer study while subsequent reports will present the findings in a more detailed manner.

Materials and Methods.—Inoculum: The 898 strain of Eaton agent was recovered in monkey kidney tissue culture from a patient with nonbacterial pneumonia.[5] The tenth- and fourteenth-day fluid harvests from the second monkey kidney tissue culture passage were pooled and distributed in aliquots which were stored in glass sealed ampoules at −60°C. The pooled material contained 640 egg infectious doses per ml as determined by amniotic inoculation of 13-day embryonated eggs and immunofluorescent examination of surviving embryos after 6 days' incubation at 35°C. The inoculum was safety tested as previously described in monkey kidney, HEp-2, and rabbit kidney tissue culture as well as in rabbits, guinea pigs, and suckling and weanling mice.[6]

The inoculum was found to contain a small quantity (10TCID50 per ml) of SV40 virus, a common contaminant of rhesus monkey kidney tissue culture.[7] This virus was neutralized by specific antiserum when the inoculum was administered to the volunteers.

Recovery of Eaton agent: Attempts to recover the agent from the volunteers were made using monkey and human kidney tissue culture as described previously.[5] The tissue culture medium consisted of Eagle's basal medium and 5% inactivated chicken serum with penicillin (1,000 units/ml) as the only antibiotic. Fourteen- and 21-day culture fluids were inoculated into 13-day eggs which were incubated for 6 days at 35°C and then tested for the presence of specific antigen in the bronchial epithelium by the indirect fluorescent antibody method.

Serologic techniques: The fluorescent antibody technique was performed as described previously.[3] Cold agglutinins and Streptococcus MG agglutinins were measured by the method of Hilleman and Feller.[8]

Volunteers: An approximately equal number of men with or without detectable fluorescent stainable antibody were selected for study. These healthy adults (21–36 years of age) from the Federal prison system were isolated for 1 week before they were given 2 ml of a 1:2 dilution of tissue culture fluid containing strain 898 by coarse spray and instillation into the nose and mouth. Simultaneous titration of the final inoculum in eggs indicated that the men received approximately 640 egg infectious doses of Eaton agent. The technique whereby the men were isolated and observed for illness has been described previously.[6] Clinical examinations were performed twice daily by physicians who were unaware of the volunteers' pre-inoculation Eaton antibody status.

Results.—A total of 52 men received infectious tissue culture fluid. As seen in Table 1, all volunteers who did not possess fluorescent stainable antibody prior to

TABLE 1

INFECTION OF VOLUNTEERS WITH EATON AGENT*

Fluorescent antibody titer prior to challenge	Type of illness	No. of volunteers	Fourfold or Greater Rise in Antibody** Eaton (fluorescent)	Cold agglutinin	Strep. MG agglutinin
less than 1:10	Pneumonia†	3	3	3	1
	Otitis media‡	11	11	7	2
	Febrile upper respir. illness	2	2	1	0
	Afebrile upper respir. illness	4	4	1	0
	None	7	7	0	0
	Total	27	27	12	3
1:10 or greater	Otitis media	1	1	0	0
	Afebrile upper respir. illness	6	4	0	0
	None	18	12	0	0
	Total	25	17	0	0

* A febrile upper respiratory illness preceded the occurrence of pneumonia, otitis media, or febrile upper respiratory illness.
† One patient with pneumonia also had otitis media.
‡ Two patients with otitis media developed fever (38°C or >).
** Post challenge sera were collected on the 23rd or 24th day.

challenge developed that antibody 23 to 24 days after the agent was administered. Similarly, the majority of volunteers who had detectable antibody prior to challenge developed a rise in antibody. These findings suggest that the second tissue culture passage Eaton agent infected all seronegative volunteers and a majority of those individuals who possessed antibody prior to challenge. Isolation studies, although incomplete at present, also indicated a high rate of infection. The agent was not recovered from 21 men on the day after instillation, whereas it was recovered from 15 of these volunteers at some time between the 3rd and 13th day following inoculation.

Twenty of the seronegative volunteers and 7 of the men who had antibody prior to challenge developed mild upper respiratory illness after 4 to 9 days. Three individuals in the former group subsequently developed signs and symptoms of

pneumonia on the 9th to 13th day; fine moist rales were detected in each instance, whereas only 2 of the men became febrile (38°C or greater) and developed X-ray changes indicative of pulmonary infiltration. Four additional volunteers developed a febrile illness without evidence of pneumonia. An unanticipated finding was the occurrence of a moderately severe hemorrhagic otitis media which was characterized by the occurrence of bullous lesions of the tympanic membrane and which followed the onset of minor upper respiratory symptoms by several days. These illnesses will be described in greater detail in subsequent publications.

All but one of the 17 men who developed moderate to severe disease (pneumonia, otitis, or febrile respiratory illness) were free of detectable antibody at the time of challenge. The inverse relation between this degree of illness and pre-existing antibody suggests that the Eaton agent contained in the inoculum was responsible for the observed illnesses.

A cold agglutinin response was confined to those volunteers who were Eaton seronegative prior to challenge. Further, cold agglutinins developed predominantly in those individuals who had moderate to severe illness. Previous studies have shown that cold agglutinins and fluorescent stainable antibody are distinct and that the former correlates well with severity of illness.[1,9] As shown previously, cold agglutinins were less efficient for serodiagnosis than was the specific Eaton antibody.[3] Streptococcus MG agglutinins developed infrequently; this response was associated with one pneumonia illness and with two cases of otitis.

Discussion and Conclusions.—The findings in this study suggest that a recently isolated tissue culture strain of Eaton agent possessed the capacity to infect humans and to stimulate the production of antibody. All individuals who were free of fluorescent stainable antibody became infected and a majority developed pneumonia, otitis, or a febrile respiratory illness. Although a majority of individuals who possessed Eaton antibody at the time of inoculation became infected, the presence of such antibody appeared to protect against the occurrence of moderate to severe illness. This antibody-illness relationship strongly suggests that the Eaton agent itself was responsible for initiating the sequence of events which led to pneumonia, otitis, or febrile respiratory disease. Further, these data, together with the observations made during previous epidemiologic studies in which the agent was recovered from serologically positive individuals, support the specificity of the fluorescent staining reaction in patients with atypical pneumonia.[3]

Approximately 10 per cent of seronegative volunteers developed pneumonia. This proportion is somewhat greater than the estimate made for the occurrence of pneumonia in natural infection. During a study of Marine recruits, 83 per cent of whom were free of detectable antibody when they entered training, it was estimated that 30 infections occurred for each pneumonia which was recognized.[3] The present findings and the previous observations are consistent, however, when one considers that pneumonia is more apt to be recognized in a longitudinal study of volunteers than in men undergoing recruit training.

It is of some interest that mild upper respiratory illness occurred in a proportion of the volunteers who possessed prior Eaton antibody. It is probable that the illnesses in this group resulted from reinfection. Mild illness associated with reinfection has been observed with other respiratory agents (para influenza types 1 and 3 and respiratory syncytial virus) and appears to be a not uncommon oc-

currence.[10–12] Although antibody did not prevent mild upper respiratory illness associated with Eaton infection, it was effective in protecting against the more serious consequences of infection.

The frequency of otitis in experimental Eaton infection suggests the need for evaluation of this agent in naturally occurring ear disease. At present it is not possible to evaluate otitis in naturally occurring Eaton infection, since previous studies were oriented primarily towards pneumonia. The present findings suggest that otitis may be a more common manifestation of infection than pneumonia. Before this association can be established, however, it must be determined whether the ear localization observed in this study reflects the inoculation procedure or represents an inherent property of the agent.

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*NOTE ON HYDROMAGNETIC WAVES IN A COMPRESSIBLE FLUID CONDUCTOR**

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1. *Introduction.*—Some very interesting papers have recently been published on hydromagnetic waves. Grad[1] noticed some special solutions of great significance of the linearized hydromagnetic equations when compressibility is taken into account (all dissipative effects being neglected). If the anisotropic nature of motion of an incompressible, inviscid, and perfectly conducting medium of infinite extent embedded in a uniform magnetic field is strongly manifested by undistorted propagation of waves in one direction only—along magnetic lines of force—the compressibility acts in an even stranger manner. This becomes immediately apparent when one specifies the disturbance by means of vorticity and current density; then, the longitudinal components only of both vorticity and current density propagate along magnetic lines of force at the Alfvén velocity $A_0 = H_0\sqrt{\mu_0/4\pi\rho_0}$, H_0 being of the magnitude of the magnetic field $\mathbf{H}_0$ and μ_0 and ρ_0 the permeability and the density of the medium in the unperturbed state (emu units). This fascinating property attracted this author's attention since he began his studies on magnetohydrodynamics. It appears in Grad's article[1] and an earlier paper[2] by the writer. Part of this theory can be found in earlier papers by Baños[3] (where a complete up-to-date bibliography on that subject is given).

We also mention Ludford's contribution[4] to the subject, but this is more related to a paper[5] on "Dissipative Effects in Magnetohydrodynamic Waves" to be published in the *Comptes Rendus*.

Scholte[6] has put forward a similar theory in order to explain giant geomagnetic pulsations such as that recorded in 1958 in Europe and analyzed by Veldkamp.[7] The observed giant pulsations, it is argued, are mainly caused by the rotational part of the primary disturbance which propagates along geomagnetic lines of force without any great loss of energy. Therefore, this theory seems to offer, at least tentatively, an explanation of ionospheric noise generation and geomagnetic pulsations and may apply to radiations from natural and artificial sources, but there is, of course, considerably more study effort to be done in this area.

This was about the state of art at the beginning of 1960 when two striking papers by Lighthill[8] revitalized the theory and its potential applications. Certainly, the beautiful simplicity of certain wave-motion equations could not escape Lighthill's attention who, in fact, rediscovered some important features[1–3] of this theory in the course of his "Studies on Magnetohydrodynamic Waves and Other Anisotropic Motions."[8a]

The purpose of the present paper is first to improve certain results obtained in our earlier paper[2] and second, in the light of these results, to give a brief systematic account of wave-motion equations satisfied by the rate-of-deformation e_{ij} and a new pseudo-tensor γ_{ij}, the latter being derived from the magnetic field in the

same manner as the former is derived from material velocity; γ_{ij} appears somewhat coupled to e_{ij} in our hydromagnetic formulation. Lighthill[8a] was the first to give a wave equation for the e_{33} component of e_{ij} and the expansion (or first deformation invariant), while Grad[1] gave similar equations (coupled) for expansion, density, and longitudinal components of material velocity and magnetic induction. But up to now, there has been no systematic attempt to formulate wave-motion equations for e_{ij} and γ_{ij}. There are precisely such equations that I will derive in Section 6.

2. *Fundamental Equations.*—We begin with the following linearized hydromagnetic equations, which, neglecting all dissipative effects, are

$$\rho_0 \frac{\partial \mathbf{v}}{\partial t} = -\text{grad } \phi + \frac{\mu_0 H_0}{4\pi} \frac{\partial \mathbf{h}}{\partial z}, \tag{1}$$

$$\frac{\partial \mathbf{h}}{\partial t} = H_0 \frac{\partial \mathbf{v}}{\partial z} - \mathbf{H}_0 \text{ div } \mathbf{v}, \tag{2}$$

$$\frac{\partial \rho}{\partial t} + \rho_0 \text{ div } \mathbf{v} = 0, \tag{3}$$

$$\text{div } \mathbf{h} = 0, \tag{4}$$

where the z-axis is taken in the direction of field $\mathbf{H}_0$, ρ, $\mathbf{v}$, $\mathbf{h}$ denote the perturbations in density ρ_0, $\mathbf{v}_0 = 0$, $\mathbf{H}_0$, and $\phi = a_0^2\rho + (\mu_0 H_0 h_z)/4\pi$, where a_0 is the ordinary sound speed in the unperturbed state.

Taking the curl of equations (1) and (2), we have[2]

$$2\rho_0 \frac{\partial \omega}{\partial t} = \mu_0 H_0 \frac{\partial \mathbf{j}}{\partial z}, \tag{5}$$

$$4\pi \frac{\partial \mathbf{j}}{\partial t} = 2H_0 \frac{\partial \omega}{\partial z} - \frac{\mathbf{H}_0}{\rho_0} \times \text{grad } \frac{\partial \rho}{\partial t}, \tag{6}$$

where $\omega = (1/2)$ curl $\mathbf{v}$ is the vorticity, and $\mathbf{j} = (1/4\pi)$ curl $\mathbf{h}$ is the current density.

It follows that vorticity components ω_x, ω_y, and ω_z and current-density components j_x, j_y, and j_z satisfy the following wave-motion equations:[2]

$$\frac{\partial^2 \omega_x}{\partial t^2} - A_0^2 \frac{\partial^2 \omega_x}{\partial z^2} = \frac{A_0^2}{2\rho_0} \frac{\partial^3 \rho}{\partial t \partial y \partial z},$$

$$\frac{\partial^2 \omega_y}{\partial t^2} - A_0^2 \frac{\partial^2 \omega_y}{\partial z^2} = -\frac{A_0^2}{2\rho_0} \frac{\partial^3 \rho}{\partial t \partial x \partial z}, \tag{7}$$

$$\frac{\partial^2 \omega_z}{\partial t^2} - A_0^2 \frac{\partial^2 \omega_z}{\partial z^2} = 0, \tag{8}$$

and

$$\frac{\partial^2 j_x}{\partial t^2} - A_0^2 \frac{\partial^2 j_x}{\partial z^2} = \frac{H_0}{4\pi\rho_0} \frac{\partial^3 \rho}{\partial t^2 \partial y},$$

$$\frac{\partial^2 j_y}{\partial t^2} - A_0^2 \frac{\partial^2 j_y}{\partial z^2} = -\frac{H_0}{4\pi\rho_0} \frac{\partial^3 \rho}{\partial t^2 \partial x}, \tag{9}$$

$$\frac{\partial^2 j_z}{\partial t^2} - A_0^2 \frac{\partial^2 j_z}{\partial z^2} = 0, \tag{10}$$

where

$$j_z = \pm \frac{H_0}{2\pi A_0} \omega_z. \tag{11}$$

Therefore, it appears that while the transverse components are coupled to the density oscillations and are convected with the fluid, the longitudinal components ignore these density oscillations and are propagated along lines of force (in both directions) without attenuation.

The coupling relationship (11) between longitudinal components shows that

(*a*) it does not depend on the magnitude of the magnetic field present;
(*b*) the vanishing of either component involves the vanishing of the other; this occurs when either quantity is zero initially.

The density satisfies[1,2]

$$\frac{\partial^2 \rho}{\partial t^2} = a_0^2 \nabla^2 \rho + \frac{A_0^2 \rho_0}{H_0} \nabla^2 h_z = a_0^2 \nabla^2 \rho + \frac{4\pi A_0^2 \rho_0}{H_0} \left(\frac{\partial j_x}{\partial y} - \frac{\partial j_y}{\partial x} \right), \tag{12}$$

where

$$\nabla^2 = \partial^2/\partial x^2 + \partial^2/\partial y^2 + \partial^2/\partial z^2.$$

3. *Wave-Motion Equations for the Density and Transverse Components.*—These results can be improved as follows: First, elimination of j_x and j_y between equations (9) and (12) yields

$$\frac{\partial^2}{\partial t^2} \left(\frac{\partial^2 \rho}{\partial t^2} - a_0^2 \nabla^2 \rho \right) - A_0^2 \frac{\partial^2}{\partial z^2} \left(\frac{\partial^2 \rho}{\partial t^2} - a_0^2 \nabla^2 \rho \right) = A_0^2 \frac{\partial^2}{\partial t^2} \left(\frac{\partial^2 \rho}{\partial x^2} + \frac{\partial^2 \rho}{\partial y^2} \right), \tag{13}$$

where a two-dimensional Laplacian appears. This equation shows plainly the radical departure of the "new sound-wave equation" from the ordinary sound-wave equation in the absence of a magnetic field. It can be used to actually evaluate ρ by successive approximations (Picard reiteration method). Equation (13) can be rewritten

$$\frac{\partial^2}{\partial t^2} \left(\frac{\partial^2 \rho}{\partial t^2} - (a_0^2 + A_0^2) \nabla^2 \rho \right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2} \nabla^2 \rho = 0. \tag{14}$$

Differentiation with respect to t of terms of equation (14) yields an equation given by Lighthill[3a] for the expansion $\Delta = \operatorname{div} \mathbf{v}$. For time harmonic dependence, one finds a result given earlier by Baños (see reference 3b, p. 352).

On the other hand, elimination of ρ between equations (7) and (14) gives

$$\left\{ \frac{\partial^2}{\partial t^2} \left[\frac{\partial^2}{\partial t^2} \left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2) \nabla^2 \right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2} \nabla^2 \right] - A_0^2 \frac{\partial^2}{\partial z^2} \left[\frac{\partial^2}{\partial t^2} \left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2) \nabla^2 \right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2} \nabla^2 \right] \right\}_{\omega_y}^{\omega_z} = 0. \tag{15}$$

Similarly, elimination of ρ between equations (9) and (14) gives

$$\left\{\frac{\partial^2}{\partial t^2}\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\right] - A_0^2 \frac{\partial^2}{\partial z^2}\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\right]\right\}_{j_y}^{j_x} = 0. \quad (16)$$

Equations (15) and (16) show that the quantities

$$\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\right]_{j_x, j_y}^{\omega_x, \omega_y} \quad (17)$$

are propagated along magnetic lines of force at Alfvén velocity A_0. These quantities are identically zero if they are zero initially; under this condition, equations (15) and (16) reduce to fourth-order equations of the same type as equation (14).

4. *Coupling Relationship between Vorticity and Current Density.*—Following a suggestion due to C. Walén (personal communication), we can put

$$\mathbf{j} = \pm \frac{H_0}{2\pi A_0}\omega + \mathbf{H}_0 \times \text{grad } \varphi, \quad (18)$$

for div ($\mathbf{H}_0 \times$ grad φ) is identically zero and relation (11) is satisfied. To determine φ, we substitute (18) into equations (5) and (6). This gives

$$\mathbf{H}_0 \times \text{grad}\left(\frac{\partial \varphi}{\partial t} \pm A_0 \frac{\partial \varphi}{\partial z} + \frac{1}{4\pi\rho_0}\frac{\partial \rho}{\partial t}\right) = 0. \quad (19)$$

Hence, φ satisfies

$$\frac{\partial \varphi}{\partial t} \pm A_0 \frac{\partial \varphi}{\partial z} + \frac{1}{4\pi\rho_0}\frac{\partial \rho}{\partial t} = 0. \quad (20)$$

Elimination of ρ between equations (14) and (20) gives

$$\frac{\partial}{\partial t}\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2 \varphi}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\varphi\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\varphi\right] \pm A_0 \frac{\partial}{\partial z}\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2 \varphi}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\varphi\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\varphi\right] = 0, \quad (21)$$

as one would have expected by virtue of equations (15) and (16).

5. *A New Pseudo-Tensor, γ_{ij}, Derived from Magnetic Field.*—By the way of digression, we now introduce the quantity

$$\gamma_{ij} = \frac{1}{2}\left(\frac{\partial h_i}{\partial x_j} + \frac{\partial h_j}{\partial x_i}\right), \quad (22)$$

where for convenience usual tensor notations are employed. This is obviously mathematically derived from the magnetic field in the same way as the rate-of-deformation

$$e_{ij} = \frac{1}{2}\left(\frac{\partial v_i}{\partial x_j} + \frac{\partial v_j}{\partial x_i}\right) \quad (23)$$

is derived from material velocity. γ_{ij} is somewhat coupled to e_{ij} by our hydro-

magnetic formulation, equations (1)–(4), as will shortly appear. Its physical significance is not obvious (it has dimensions of current density), but, following an analysis for material velocity due to Stokes,[9] we can say this: if one compares, at the time t, the magnetic fields at two infinitely near material particles at x_i and $x'_i = x_i + \delta x_i$, then one has

$$h'_i = h_i + \frac{\partial h_i}{\partial x_j}\delta x_j, \tag{24}$$

which can be written as

$$h'_i = h_i + \frac{1}{2}\left(\frac{\partial h_i}{\partial x_j} - \frac{\partial h_j}{\partial x_i}\right)\delta x_j + \frac{1}{2}\left(\frac{\partial h_i}{\partial x_j} + \frac{\partial h_j}{\partial x_i}\right)\delta x_j \tag{25}$$

or, in vector notations,

$$\mathbf{h}' = \mathbf{h} + 2\pi\mathbf{j} \times \delta\mathbf{r} + (1/2)\,\mathrm{Grad}_{\delta x_i} F, \tag{26}$$

where we set

$$F = h_{ij}\delta x_i \delta x_j, \tag{27}$$

and the gradient is taken with respect to δx_i (x_i being fixed). Now, while the second term on the right side of equation (26) expresses plainly Laplace's law, the last part is somewhat more difficult to interpret and is arrested here for further investigation. With these preliminaries, we pass to the last section of this paper and give wave equations for both e_{ij} and γ_{ij}.

6. *Wave-Motion Equations for e_{ij} and γ_{ij}.*—When e_{ij} and γ_{ij} are introduced in equations (1) and (2), these become

$$\left.\begin{aligned}
\rho_0\frac{\partial e_{11}}{\partial t} &= -a_0^2\frac{\partial^2\rho}{\partial x^2} + \mu_0 H_0\frac{\partial j_y}{\partial x},\\
\rho_0\frac{\partial e_{22}}{\partial t} &= -a_0^2\frac{\partial^2\rho}{\partial y^2} - \mu_0 H_0\frac{\partial j_x}{\partial y},\\
\rho_0\frac{\partial e_{33}}{\partial t} &= -a_0^2\frac{\partial^2\rho}{\partial z^2},\\
\rho_0\frac{\partial e_{23}}{\partial t} &= -a_0^2\frac{\partial^2\rho}{\partial y\partial z} - \frac{\mu_0 H_0}{2}\frac{\partial j_x}{\partial z},\\
\rho_0\frac{\partial e_{31}}{\partial t} &= -a_0^2\frac{\partial^2\rho}{\partial z\partial x} + \frac{\mu_0 H_0}{2}\frac{\partial j_y}{\partial z},\\
\rho_0\frac{\partial e_{12}}{\partial t} &= -a_0^2\frac{\partial^2\rho}{\partial x\partial y} + \frac{\mu_0 H_0}{2}\left(\frac{\partial j_y}{\partial y} - \frac{\partial j_x}{\partial x}\right),
\end{aligned}\right\} \tag{28}$$

These equations are highly unsymmetrical and show perhaps more plainly than equations (1) and (2) the strongly anisotropic character of the electrically conducting fluid. However, using the results obtained in Section 3, the conclusions to be drawn are surprisingly simple. First, it follows at once by virtue of equation (14), that e_{33} satisfies

and

$$\left.\begin{aligned}
\frac{\partial \gamma_{11}}{\partial t} &= H_0 \frac{\partial e_{11}}{\partial z}, \\
\frac{\partial \gamma_{22}}{\partial t} &= H_0 \frac{\partial e_{22}}{\partial z}, \\
\frac{\partial \gamma_{33}}{\partial t} &= \frac{H_0}{\rho_0} \frac{\partial^2 \rho}{\partial t \partial z} + H_0 \frac{\partial e_{33}}{\partial z}, \\
\frac{\partial \gamma_{23}}{\partial t} &= \frac{H_0}{2\rho_0} \frac{\partial^2 \rho}{\partial t \partial y} + H_0 \frac{\partial e_{23}}{\partial z}, \\
\frac{\partial \gamma_{31}}{\partial t} &= \frac{H_0}{2\rho_0} \frac{\partial^2 \rho}{\partial t \partial x} + H_0 \frac{\partial e_{31}}{\partial z}, \\
\frac{\partial \gamma_{12}}{\partial t} &= H_0 \frac{\partial e_{12}}{\partial z}.
\end{aligned}\right\} \tag{29}$$

$$\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2 e_{33}}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2 e_{33}\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2 e_{33} = 0, \tag{30}$$

a result obtained earlier by Lighthill.[8a] We may add that the same equation is also satisfied by γ_{33}, as seen by inspection of the third equation in (29).

By successive eliminations, we then find that all other components satisfy a unique sixth-order differential equation of the type as that encountered in Section 3; therefore, we have

$$\left\{\frac{\partial^2}{\partial t^2}\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\right] - A_0^2 \frac{\partial^2}{\partial z^2}\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\right]\right\}_{\gamma'_{ij}}^{e'_{ij}} = 0, \tag{31}$$

where we use the prime in order to exclude the values $i = j = 3$ of suffixes; for $i = j = 3$, we have equation (30).

If one puts

$$\begin{matrix} e'_{ij} \\ \gamma'_{ij} \end{matrix} = \begin{matrix} (e'_{ij})_0 \\ (\gamma'_{ij})_0 \end{matrix} \exp[i(\omega t - \alpha x - \beta y - \gamma z)], \tag{32}$$

one has

$$(\omega^2 - A_0^2\gamma^2)[(\omega^2 - a_0^2\gamma^2)(\omega^2 - A_0^2\gamma^2) - (\alpha^2 + \beta^2)((a_0^2 + A_0^2)\omega^2 - a_0^2 A_0^2 \gamma^2)] = 0. \tag{33}$$

Hence: Either

(*a*) $\gamma = \pm\omega/A_0$ and then e'_{ij}, γ'_{ij} are propagated solely along magnetic lines of force without attenuation at the Alfvén velocity A_0; or

(*b*) $\gamma \neq \pm\omega/A_0$, and then

$$\alpha^2 + \beta^2 = \frac{(\omega^2 - a_0^2\gamma^2)(\omega^2 - A_0^2\gamma^2)}{(a_0^2 + A_0^2)\omega^2 - a_0^2 A_0^2 \gamma^2}, \tag{34}$$

which represents the wave number surface given by Lighthill.[8]

Under (*b*), we distinguish two subcases:

(*b*.1) $\gamma = \pm\omega/a_0$, and then e'_{ij}, γ'_{ij} are propagated one-dimensionally, along magnetic lines of force, at the sound speed a_0; in this case, $\alpha = \beta = 0$, that is, the ovoid sheet of (34) collapses into the γ-axis.

(*b*.2) $\gamma \neq \pm\omega/a_0$; by rewriting equation (34) as follows,

$$\alpha^2 + \beta^2 = \frac{1}{A_0^2} \frac{(\omega^2 - a_0^2\gamma^2)(\omega^2 - A_0^2\gamma^2)}{(1 + a_0^2/A_0^2)\omega^2 - a_0^2\gamma^2}, \tag{35}$$

or

$$\alpha^2 + \beta^2 = \frac{1}{a_0^2} \frac{(\omega^2 - a_0^2\gamma^2)(\omega^2 - A_0^2\gamma^2)}{(1 + A_0^2/a_0^2)\omega^2 - A_0^2\gamma^2}, \tag{36}$$

two limiting cases, considered in slightly different forms by Baños,[3b] Grad,[1] and Lighthill,[8] are readily revealed:

(*b*.2.1) $A_0 \gg a_0$, when equation (35) reduces approximately to

$$\alpha^2 + \beta^2 + \gamma^2 = \frac{\omega^2}{A_0^2}, \tag{37}$$

and we have propagation in *all* directions with nearly equal velocity A_0;

(*b*.2.2) $A_0 \ll a_0$, when equation (36) reduces approximately to

$$\alpha^2 + \beta^2 + \gamma^2 = \frac{\omega^2}{a_0^2}, \tag{38}$$

and we have isotropic tri-dimensional propagation with velocity nearly a_0.

The singular case $\gamma = \pm\omega\sqrt{a_0^{-2} + A_0^{-2}}$ when $\alpha^2 + \beta^2 = \infty$, requires more care and is omitted here.

Appendix.—The wave-motion equations for e_{ij} and γ_{ij} may also be derived by the following brief analysis, which re-emphasizes the compressibility effect of discrimination between longitudinal and transverse components.

The z-component of material velocity satisfies

$$\rho_0 \frac{\partial v_z}{\partial t} = -a_0^2 \frac{\partial \rho}{\partial z}. \tag{A.1}$$

Hence, by virtue of equation (14), we have at once

$$\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\right] v_z = 0. \tag{A.2}$$

On the other hand,

$$\frac{\partial h_z}{\partial t} = H_0 \frac{\partial v_z}{\partial z} - H_0 \operatorname{div} \mathbf{v}. \tag{A.3}$$

Since both v_z and div $\mathbf{v}$ satisfy (A.2), it follows that

$$\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\right] h_z = 0. \tag{A.4}$$

Combining equations for the transverse components in (1) and (2) with equations (14), (A.2), and (A.4), we obtain

$$\left\{\frac{\partial^2}{\partial t^2}\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\right] - A_0^2 \frac{\partial^2}{\partial z^2}\left[\frac{\partial^2}{\partial t^2}\left(\frac{\partial^2}{\partial t^2} - (a_0^2 + A_0^2)\nabla^2\right) + a_0^2 A_0^2 \frac{\partial^2}{\partial z^2}\nabla^2\right]\right\}_{h_x, h_y}^{v_x, v_y} = 0. \tag{A.5}$$

The wave-motion equations for e_{ij} and γ_{ij} become obvious. Furthermore, the propagation of transverse divergence

$$\delta = \frac{\partial v_x}{\partial x} + \frac{\partial v_y}{\partial y}, \tag{A.6}$$

as well as that of transverse divergence of magnetic field, also becomes apparent. According to Lighthill (see reference 8b, p. 467), the propagation of v_z and δ, governed by equations (A.2) and (A.5) respectively, are important for waves through gases at pressures small compared with magnetic pressure.

The author wishes to express here his appreciation to Professors T. G. Cowling and H. Grad and Dr. C. Walén for their comments on his earlier paper.[2] Thanks are due to Dr. R. V. Row of this laboratory, who read the manuscript and made suggestions for its improvement. The results given here were presented at the New York University Institute of Mathematical Sciences, Magneto-Fluid Dynamics Seminar, on March 8, 1961. The author takes this opportunity to thank Professor J. Berkowitz, Acting Director of the Magneto-Fluid Dynamics Division, for his interest and courtesy.

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ERRATA

In the paper entitled "Assembly of the Peptide Chains of Hemoglobin," by Howard M. Dintzis, which appeared in volume 47, number 3, pages 247–261, the author wishes to make the following correction:

On page 251, five lines below Figure 2, the phrase "and 2 *M* formic acid—0.02 *M* pyridine" should read "and 2 *M* formic acid—0.2 *M* pyridine."

In the paper entitled "The Thermostatic Control of Human Metabolic Heat Production," by T. H. Benzinger, A. W. Pratt, and Charlotte Kitzinger, which appeared in the May issue of volume 47, where the caption for Figure 3, p. 735, reads "*internal*-sensory receptor site, *D*," the "*D*" should read "*A*." Throughout the remainder of the caption, for "*D*" read "*A*."

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